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(54) Title: OB PROTEIN DERIVATIVES HAVING PROLONGED HALF-LIFE		
(57) Abstract The present invention concerns long half-life derivative of the obesity protein OB. The invention specifically concerns OB protein-immunoglobulin chimeras and polyethylene glycol (PEG)-OB derivatives, which have extended half-life as compared to the corresponding native OB proteins. The invention further relates to methods for appetite and/or weight reduction and for treating other physiological conditions by using the long half-life derivatives of OB.		

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OB PROTEIN DERIVATIVES HAVING PROLONGED HALF-LIFE

Field of the Invention

The invention concerns long half-life derivatives of the OB protein. In particular, the invention concerns OB protein-immunoglobulin chimeras, and other long half-life derivatives of the OB protein, and compositions comprising and methods for administering them. The invention further relates to a method for treating obesity by administering a long half-life variant of the OB protein, such as, an OB protein-immunoglobulin chimera.

Background of the Invention

Obesity is the most common nutritional disorder which, according to recent epidemiologic studies, affects about one third of all Americans 20 years of age or older. Kuczmarski *et al.*, J. Am. Med. Assoc. 272, 205-11 (1994). Obesity is responsible for a variety of serious health problems, including cardiovascular disorders, type II diabetes, insulin-resistance, hypertension, hypertriglyceridemia, dyslipoproteinemia, and some forms of cancer. Pi-Sunyer, F.X., Anns. Int. Med. 119, 655-60 (1993); Colfitz, G.A., Am. J. Clin. Nutr. 55, 503S-507S (1992). A single-gene mutation (the obesity or "ob" mutation) has been shown to result in obesity and type II diabetes in mice. Friedman, Genomics 11, 1054-1062 (1991). Zhang *et al.*, Nature 372, 425-431 (1994) have recently reported the cloning and sequencing of the mouse *ob* gene and its human homologue, and suggested that the *ob* gene product may function as part of a signalling pathway from adipose tissue that acts to regulate the size of the body fat depot. Parabiosis experiments performed more than 20 years ago predicted that the genetically obese mouse containing two mutant copies of the *ob* gene (*ob/ob* mouse) does not produce a satiety factor which regulates its food intake, while the diabetic (*db/db*) mouse produces but does not respond to a satiety factor. Coleman and Hummel, Am. J. Physiol. 217, 1298-1304 (1969); Coleman, Diabetol 2, 294-98 (1973). Recent reports by three independent research teams have demonstrated that daily injections of recombinant OB protein inhibit food intake and reduce body weight and fat in grossly obese *ob/ob* mice but not in *db/db* mice (Pellemounter *et al.*, Science 269, 540-43 [1995]; Halaas *et al.*, Science 269, 543-46 [1995]; Campfield *et al.*, Science 269, 546-49 [1995]), suggesting that the *ob* protein is such a satiety factor as proposed in early cross-circulation studies. The results of these first studies leave many questions unanswered, and show a number of as yet unresolved discrepancies. For example, while modest effects of daily injections of the *ob* protein on food intake and body weight were reported in lean mice, there was a significant reduction in body fat as assessed by carcass composition in one (Halaas *et al.*, *supra*) but not in another (Pellemounter *et al.*, *supra*) of these reports, despite equivalent decreases in body weight. Furthermore, Pellemounter *et al.*, *supra* observed that, for reasons unknown, *ob/ob* mice treated with a 0.1 mg/kg/day dose of the OB protein actually increased their body weight by 17.13 %, while the weight reduction in the obese mice that received a 1 mg/kg/day dose of *ob* was rather moderate. The receptor or receptors of the *ob* protein are as of yet unidentified. While the existence of peripheral receptors cannot be ruled out at this time, the recent report that an increased

- expression of the *ob* gene in adipose tissue of mice with hypothalamic lesions does not result in a lean phenotype suggests that the OB protein does not act directly on fat cells. Maffei *et al.*, *Proc. Natl. Acad. Sci.* **92**, 6957-60 (1995). Researchers suggest that at least one OB receptor is localized in the brain. The identification and expression cloning of a leptin receptor (OB-R) was reported by Tartaglia *et al.*, *Cell* **83**, 1263-71 (1995).
- 5 Various isoforms of a leptin receptor are described by Cioffi *et al.*, *Nature* **2**, 585-89 (1996). A human hematopoietin receptor, which might be a receptor of the OB protein, is described in PCT application Publication No. WO 96/08510, published 21 March 1996. A receptor of the OB protein is disclosed in Tartaglia *et al.*, *Cell* **83**, 1263-71 (1995).

Summary of the Invention

- 10 The present invention is based on the observation that the OB protein is significantly more effective at reducing body weight and adipose tissue weight when delivered as a continuous subcutaneous infusion than when the same dose is delivered as a daily subcutaneous injection. The invention is further based on the unexpected finding that a chimeric protein, in which the OB polypeptide is fused to an immunoglobulin constant domain, is strikingly more potent in reducing the body weight and adipose depots than native human
- 15 OB, when both proteins are administered by subcutaneous injection once a day. The latter observation is particularly surprising since the OB protein-immunoglobulin chimera due to its large molecular weight, is not expected to be able to cross the blood-brain barrier, and reach the OB receptor which has been believed to be located in the brain.

- In one aspect, the invention concerns long half-life derivatives of an OB protein capable of reducing
- 20 body weight and/or food intake in an individual treated. The invention further concerns compositions containing such derivatives, and their administration for reducing body weight and/or food intake.

- In another aspect, the invention concerns chimeric polypeptides comprising an OB protein amino acid sequence capable of binding to a native OB receptor linked to an immunoglobulin sequence (briefly referred to as OB-immunoglobulin chimeras or immunoadhesins). In a specific embodiment, the chimeric polypeptides
- 25 comprise a fusion of an OB amino acid sequence capable of binding a native OB receptor, to an immunoglobulin constant domain sequence. The OB portion of the chimeras of the present invention preferably has sufficient amino acid sequences from a native OB protein to retain the ability to bind to and signal through a native OB receptor. Most preferably, the OB protein retains the ability to reduce body weight when administered to obese human or non-human subjects. The OB polypeptide is preferably human, and the fusion is preferably with an
- 30 immunoglobulin heavy chain constant domain sequence. In a particular embodiment, the association of two OB polypeptide-immunoglobulin heavy chain fusions (e.g., via covalent linkage by disulfide bond(s)) results in a homodimeric immunoglobulin-like structure. An immunoglobulin light chain may further be associated with one or both of the OB-immunoglobulin chimeras in the disulfide-bonded dimer to yield a homotrimeric or homotetrameric structure.

- 35 The invention further concerns nucleic acid encoding chimeric polypeptide chains of the present invention, expression vectors containing DNA encoding such molecules, transformed host cells, and methods for the production of the molecules by cultivating transformant host cells.

Although the long half-life derivatives of the present invention are particularly useful for reducing body weight and/or food intake, they can generally be used for the treatment of conditions associated with the abnormal expression or function of the OB gene and/or to elicit biological responses mediated by an OB receptor. Thus, the OB derivatives of the present invention may be used to treat bulimia, to reduce insulin levels, e.g. in Type I or II diabetic patients, and as mitogens of various cell types expressing an OB receptor. All these and related uses are within the scope of the present invention.

In another embodiment, the invention concerns the purification of an OB receptor by using an OB protein-immunoglobulin chimera.

Brief Description of the Figures

Figure 1 top -- Lean female mice were treated with murine OB protein either as a continuous subcutaneous infusion or daily subcutaneous injections. The data shown are the mean body weight of each group, in grams, $n = 4$ mice/point.

Figure 1 bottom -- The mean weight of the retroperitoneal fat pads are shown. Continuous subcutaneous infusions of the OB protein were also more effective than daily subcutaneous injections at reducing adipose tissue weight.

Figure 2 top -- Obese female *ob/ob* mice were treated with human OB protein (hOB) or with a human OB-IgG-1 fusion protein (hOB-IgG-1). The data shown are the mean change in body weight for each treatment group from the first to the last day of experiment, in grams, $n = 3$ mice/bar except for the hOB 0.19 mg/kg/day by injection group, where $n = 4$, and PBS injection group, where $n = 1$.

Figure 2 bottom -- The data shown were the mean food intake for each treatment group for the six 24 hour periods of the experiment, in grams/mouse/day, $n = 1$ /bar.

Figure 3 top and bottom -- Obese (*ob/ob*) female mice were treated with either hOB or the hOB-IgG-1 fusion protein by daily subcutaneous injections for 7 days. The data are depicted as in Figure 2, with $n = 4$ for all treatment groups.

Figure 4 top -- Obese female *ob/ob* mice were treated with human protein (hOB) or with PEG-hOB. The data shown are the mean change in body weight for each treatment group from the first to the last day of experiment, in grams, $n = 3-4$ mice/bar except for the PBS injection group, where $n = 1$. The materials were injected daily subcutaneously. The "PEG 1X" and "PEG 2X" refer to the ratio of the PEG reagent to protein in the preparation of the molecule.

Figure 4 bottom -- The data shown were the mean food intake for each treatment group for the six 24 hour periods of the experiment, in grams/mouse/day, $n = 3-4$ /bar.

Figure 5 -- Obese (*ob/ob*) female mice were treated with either the hOB-IgG fusion protein, native hOB, or hCD4-IgG by daily subcutaneous injections for 7 days. $n = 6$ for all treatment groups, except hOB at 3.8 mg/kg/d, where $n = 2$. Again it was observed that the fusion protein was more effective than the native hOB protein at reducing body weight (top and middle panels) and food intake (bottom panel).

Figure 6 -- The nucleotide sequence (SEQ. ID. NO:1) and the amino acid sequence (SEQ. ID. NO: 2) of the human OB-IgG-1 chimera of Example 1.

Detailed Description of the Invention

A. Definitions

The term "obesity" is used to designate a condition of being overweight associated with excessive bodily fat. The desirable weight for a certain individual depends on a number of factors including sex, height, age, overall built, etc. The same factors will determine when an individual is considered obese. The determination of an optimum body weight for a given individual is well within the skill of an ordinary physician.

The phrase "long half-life" and grammatical variants thereof, as used in connection with OB derivatives, concerns OB derivatives having a longer plasma half-life and/or slower clearance than a corresponding native OB protein. The long half-life derivatives preferably will have a half-life at least about 1.5-times longer than a native OB protein; more preferably at least about 2-times longer than a native OB protein, more preferably at least about 3-time longer than a native OB protein. The native OB protein preferably is that of the individual to be treated.

The terms "OB", "OB polypeptide", "OB protein" and their grammatical variants are used interchangeably and refer to "native" or "native sequence" OB proteins (also known as "leptins") and their functional derivatives. The OB polypeptides have the typical structural features of cytokines, i.e. polypeptides released by one cell population which act on another cell as intercellular mediators, such as, for example, growth hormones, insulin-like growth factors, interleukins, insulin, glycoprotein hormones such as, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), tumor necrosis factor- α and - β (TNF- α and - β), nerve growth factors, such as NGF- β , PDGF, transforming growth factors (TGFs) such as, TGF- α and TGF- β , insulin-like growth factor-1 and -2 (IGF-1 and IGF-2), erythropoietin, osteoinductive factors, interferons (IFNs) such as, IFN- α , IFN- β and IFN- γ , colony stimulating factors (CSFs) such as, M-CSF, GM-CSF, and G-CSF, interleukins (ILs) such as, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 and other polypeptide factors.

The terms "native" and "native sequence" OB polypeptide are used to refer to an OB polypeptide from any animal species (e.g. human, murine, rabbit, cat, cow, sheep, chicken, porcine, equine, etc.), as occurring in nature, including naturally-occurring alleles, deletion, substitution and/or insertion variants, as currently known or as might be identified in the future, provided that they retain the ability to bind to and, preferably, signal through the OB receptor. Thus, a native human OB polypeptide includes the amino acid sequence between the N-terminus and the cysteine (Cys) at position 167 of the amino acid sequence shown in Figure 6 (see also SEQ. ID. NO: 2 and Figure 6 of Zhang *et al.*, *supra*), and naturally occurring variants of this protein, as currently known or might be identified in the future. Similarly, a "native" or "native sequence" murine OB polypeptide has the amino acid sequence shown in Figure 6 of Zhang *et al.*, *supra*, and naturally occurring variants of that polypeptide, as currently known or might be identified in the future. The definition specifically includes variants with or without a glutamine at amino acid position 49, using the amino acid numbering of Zhang *et al.*, *supra*. The terms "native" and "native sequence" OB polypeptide include the native proteins with or without the initiating N-terminal methionine (Met), and with or without the native signal sequence, either in monomeric or in dimeric form. The native human and murine OB polypeptides known in the art are 167 amino acids long, contain two conserved cysteines, and have the features of a secreted protein. The polypeptide is largely

hydrophilic, and the predicted signal sequence cleavage site is at position 21, using the amino acid numbering of Zhang *et al.*, *supra*. The overall sequence homology of the human and murine sequences is about 84%. The two proteins show a more extensive identity in the N-terminal region of the mature protein, with only four conservative and three non-conservative substitutions among the residues between the signal sequence cleavage site and the conserved Cys at position 117. The molecular weight of OB proteins is about 16 kD in a monomeric form.

A "functional derivative" of a native polypeptide is a compound having a qualitative biological property in common with the native polypeptide. A functional derivative of an OB polypeptide is a compound that has a qualitative biological property in common with a native (human or non-human) OB polypeptide. "Functional derivatives" include, but are not limited to, fragments of native polypeptides from any animal species (including humans), and derivatives of native (human and non-human) polypeptides and their fragments, provided that they have a biological activity in common with a corresponding native polypeptide.

"Fragments" comprise regions within the sequence of a mature native OB polypeptide. Preferred fragments of OB polypeptides include the C-terminus of the mature protein, and may contain relatively short deletion(s) at the N-terminus and in other parts of the molecule not required for receptor binding and/or for structural integrity.

The term "derivative" is used to define amino acid sequence variants, and covalent modifications of a native polypeptide, whereas the term "variant" refers to amino acid sequence variants within this definition.

"Biological property" in the context of the definition of "functional derivatives" is defined as either 1) immunological cross-reactivity with at least one epitope of a native polypeptide (e.g. a native OB polypeptide of any species), or 2) the possession of at least one adhesive, regulatory or effector function qualitatively in common with a native polypeptide.

Preferably, the functional derivatives are polypeptides which have at least about 65% amino acid sequence identity, more preferably about 75% amino acid sequence identity, even more preferably at least about 85% amino acid sequence identity, most preferably at least about 95% amino acid sequence identity with a native polypeptide. In the context of the present invention, functional derivatives of native sequence human OB polypeptides preferably show at least 95% amino acid sequence identity with the native OB proteins, and are not immunogenic in the human.

Amino acid sequence identity or homology is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding native polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology.

Immunologically cross-reactive as used herein means that the candidate (poly)peptide is capable of competitively inhibiting the qualitative biological activity of a corresponding native polypeptide having this activity with polyclonal antibodies or antisera raised against the known active molecule. Such antibodies and antisera are prepared in conventional fashion by injecting an animal such as a goat or rabbit, for example,

subcutaneously with the known native OB protein in complete Freud's adjuvant, followed by booster intraperitoneal or subcutaneous injection in incomplete Freud's.

The term "isolated OB polypeptide" and grammatical variants thereof refer to OB polypeptides (as hereinabove defined) separated from contaminant polypeptides present in the human, other animal species, or
5 in other source from which the polypeptide is isolated.

In general, the term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a reference (e.g. native sequence) polypeptide. The amino acid alterations may be substitutions, insertions, deletions or any desired combinations of such changes in a native amino acid sequence.

10 Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino
15 acid at a particular position in a native amino acid sequence. Immediately adjacent to an amino acid means connected to either the α -carboxy or α -amino functional group of the amino acid.

Deletional variants are those with one or more amino acids in the native amino acid sequence removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule.

"Covalent derivatives" include modifications of a native polypeptide or a fragment thereof with an
20 organic proteinaceous or non-proteinaceous derivatizing agent, and post-translational modifications. Covalent modifications are traditionally introduced by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected sites or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl
25 and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues may be present in the OB-immunoglobulin chimeras of the present invention. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, tyrosine or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side
30 chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)].

The terms "DNA sequence encoding", "DNA encoding" and "nucleic acid encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide chain. The DNA sequence thus
35 codes for the amino acid sequence.

The terms "replicable expression vector" and "expression vector" refer to a piece of DNA, usually double-stranded, which may have inserted into it a piece of foreign DNA. Foreign DNA is defined as heterologous DNA, which is DNA not naturally found in the host cell. The vector is used to transport the

foreign or heterologous DNA into a suitable host cell. Once in the host cell, the vector can replicate independently of the host chromosomal DNA, and several copies of the vector and its inserted (foreign) DNA may be generated. In addition, the vector contains the necessary elements that permit translating the foreign DNA into a polypeptide. Many molecules of the polypeptide encoded by the foreign DNA can thus be rapidly synthesized.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancer.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or a secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

In the context of the present invention the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny. Thus, the words "transformants" and "transformed (host) cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

Native immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Clothia *et al.*, *J. Mol. Biol.* **186**, 651-663 (1985); Novotny and Haber, *Proc. Natl. Acad. Sci. USA* **82**, 4592-4596 [1985]).

Depending on the amino acid sequence of the constant region of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and

IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant regions that correspond to the different classes of immunoglobulins are called α , delta, epsilon, γ , and μ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. IgA-1 and IgA-2 are monomeric subclasses of IgA, which usually is in the form of dimers or larger polymers. Immunocytes in the gut produce mainly polymeric IgA (also referred to poly-IgA including dimers and higher polymers). Such poly-IgA contains a disulfide-linked polypeptide called the "joining" or "J" chain, and can be transported through the glandular epithelium together with the J-containing polymeric IgM (poly-IgM), comprising five subunits.

Hybridization is preferably performed under "stringent conditions" which means (1) employing low ionic strength and high temperature for washing, for example, 0.015 sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C, or (2) employing during hybridization a denaturing agent, such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6/8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μ g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

B. OB protein-immunoglobulin chimeras (immunoadhesins)

Immunoadhesins are chimeric antibody-like molecules that combine the functional domain(s) of a binding protein (usually a receptor, a cell-adhesion molecule or a ligand) with the an immunoglobulin sequence. The most common example of this type of fusion protein combines the hinge and Fc regions of an immunoglobulin (Ig) with domains of a cell-surface receptor that recognizes a specific ligand. This type of molecule is called an "immunoadhesin", because it combines "immune" and "adhesion" functions; other frequently used names are "Ig-chimera", "Ig-" or "Fc-fusion protein", or "receptor-globulin".

To date, more than fifty immunoadhesins have been reported in the art. Immunoadhesins reported in the literature include, for example, fusions of the T cell receptor (Gascoigne *et al.*, *Proc. Natl. Acad. Sci. USA* **84**, 2936-2940 [1987]); CD4 (Capon *et al.*, *Nature* **337**, 525-531 [1989]; Trautnecker *et al.*, *Nature* **339**, 68-70 [1989]; Zettmeissl *et al.*, *DNA Cell Biol. USA* **2**, 347-353 [1990]; Byrn *et al.*, *Nature* **344**, 667-670 [1990]); L-selectin (homing receptor) (Watson *et al.*, *J. Cell. Biol.* **110**, 2221-2229 [1990]; Watson *et al.*, *Nature* **342**, 164-167 [1991]); E-selectin (Mulligan *et al.*, *J. Immunol.* **151**, 6410-17 [1993]; Jacob *et al.*, *Biochemistry* **34**, 1210-1217 [1995]); P-selectin (Mulligan *et al.*, *supra*; Hollenbaugh *et al.*, *Biochemistry* **34**, 5678-84 [1995]); ICAM-1 (Stauton *et al.*, *J. Exp. Med.* **176**, 1471-1476 [1992]; Martin *et al.*, *J. Virol.* **67**, 3561-68 [1993]; Roep *et al.*, *Lancet* **343**, 1590-93 [1994]); ICAM-2 (Damle *et al.*, *J. Immunol.* **148**, 665-71 [1992]); ICAM-3 (Holness *et al.*, *J. Biol. Chem.* **270**, 877-84 [1995]); LFA-3 (Kanner *et al.*, *J. Immunol.* **148**, 2-23-29 [1992]); L1 glycoprotein (Doherty *et al.*, *Neuron* **14**, 57-66 [1995]); TNF-R1 (Ashkenazi *et al.*, *Proc. Natl. Acad. Sci. USA* **88**, 10535-539 [1991]; Lesslauer *et al.*, *Eur. J. Immunol.* **21**, 2883-86 [1991]; Peppel *et al.*, *J. Exp. Med.* **174**, 1483-1489 [1991]); TNF-R2 (Zack *et al.*, *Proc. Natl. Acad. Sci. USA* **90**, 2335-39 [1993]; Wooley *et al.*, *J.*

- Immunol.* 151, 6602-07 [1993]; CD44 [Aruffo *et al.*, *Cell* 61, 1303-1313 (1990)]; CD28 and B7 [Linsley *et al.*, *J. Exp. Med.* 173, 721-730 (1991)]; CTLA-4 [Lisley *et al.*, *J. Exp. Med.* 174, 561-569 (1991)]; CD22 [Stamenkovic *et al.*, *Cell* 66, 1133-1144 (1991)]; NP receptors [Bennett *et al.*, *J. Biol. Chem.* 266, 23060-23067 (1991)]; IgE receptor α [Ridgway and Gorman, *J. Cell. Biol.* 115, abstr. 1448 (1991)]; HGF receptor [Mark, M.R. *et al.*, 1992, *J. Biol. Chem.*, submitted]; IFN- γ α - and β -chain [Marsters *et al.*, *Proc. Natl. Acad. Sci. USA* 92, 5401-05 [1995)]; trk-A, -B, and -C [Shelton *et al.*, *J. Neurosci.* 15, 477-91 [1995)]; IL-2 (Landolfi, *J. Immunol.* 146, 915-19 [1991]); IL-10 (Zheng *et al.*, *J. Immunol.* 154, 5590-5600 [1995]).

The simplest and most straightforward immunoadhesin design combines the binding region(s) of the 'adhesin' protein with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the OB-immunoglobulin chimeras of the present invention, nucleic acid encoding the desired OB polypeptide will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible. Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of the OB-immunoglobulin chimeras.

In a preferred embodiment, the sequence of a native, mature OB polypeptide, is fused to the N-terminus of the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, e.g. IgG-1. It is possible to fuse the entire heavy chain constant region to the OB sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114 [Kobet *et al.*, *supra*], or analogous sites of other immunoglobulins) is used in the fusion. In a particularly preferred embodiment, the OB polypeptide sequence is fused to the hinge region and CH2 and CH3 or CH1, hinge, CH2 and CH3 domains of an IgG-1, IgG-2, or IgG-3 heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

In some embodiments, the OB-immunoglobulin chimeras are assembled as multimers, and particularly as homo-dimers or -tetramers (WO 91/08298). Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each four unit may be the same or different.

Various exemplary assembled OB-immunoglobulin chimeras within the scope herein are schematically diagrammed below:

- (a) AC_L-AC_L ;
- (b) $AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, \text{ or } V_LC_L-AC_H]$;
- (c) $AC_L-AC_H-[AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, \text{ or } V_LC_L-V_HC_H]$.

(d) $AC_L-V_HC_H-[AC_H]$, or $AC_L-V_HC_H$, or $V_LC_L-AC_H$;

(e) $V_LC_L-AC_H-[AC_L-V_HC_H]$, or $V_LC_L-AC_H$; and

(f) $[A-Y]_n-[V_LC_L-V_HC_H]_2$,

wherein

5 each A represents identical or different OB polypeptide amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

10 n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed as being present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the OB amino acid sequences can be inserted between immunoglobulin heavy chain and light chain sequences such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the OB polypeptide sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the CH2 domain, or between the CH2 and CH3 domains. Similar constructs have been reported by Hoogenboom, H. R. *et al.*, Mol. Immunol. **28**, 1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an OB protein-immunoglobulin heavy chain fusion polypeptide, or directly fused to the OB polypeptide. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the OB-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Method suitable for the preparation of such structures are, for example, disclosed in U.S. Patent No. 4,816,567 issued 28 March 1989.

30 In a preferred embodiment, the immunoglobulin sequences used in the construction of the immunoadhesins of the present invention are from an IgG immunoglobulin heavy chain constant domain. For human immunoadhesins, the use of human IgG-1 and IgG-3 immunoglobulin sequences is preferred. A major advantage of using IgG-1 is that IgG-1 immunoadhesins can be purified efficiently on immobilized protein A. In contrast, purification of IgG-3 requires protein G, a significantly less versatile medium. However, other structural and functional properties of immunoglobulins should be considered when choosing the Ig fusion partner for a particular immunoadhesin construction. For example, the IgG-3 hinge is longer and more flexible, so it can accommodate larger 'adhesin' domains that may not fold or function properly when fused to IgG-1. Possible IgG-based immunoadhesin structures are shown in Fig. 3a-c. While IgG immunoadhesins are typically

mono- or bivalent, other Ig subtypes like IgA and IgM may give rise to dimeric or pentameric structures, respectively, of the basic Ig homodimer unit. A typical IgM-based multimeric immunoadhesin is illustrated in Figure 3d. Multimeric immunoadhesins are advantageous in that they can bind their respective targets with greater avidity than their IgG-based counterparts. Reported examples of such structures are CD4-IgM (Traunecker *et al.*, *supra*); ICAM-IgM (Martin *et al.*, *J. Virol.* 67, 3561-68 [1993]); and CD2-IgM (Arulanandam *et al.*, *J. Exp. Med.* 177, 1439-50 [1993]).

For OB-Ig immunoadhesins, which are designed for *in vivo* application, the pharmacokinetic properties and the effector functions specified by the Fc region are important as well. Although IgG-1, IgG-2 and IgG-4 all have *in vivo* half-lives of 21 days, their relative potencies at activating the complement system are different. IgG-4 does not activate complement, and IgG-2 is significantly weaker at complement activation than IgG-1. Moreover, unlike IgG-1, IgG-2 does not bind to Fc receptors on mononuclear cells or neutrophils. While IgG-3 is optimal for complement activation, its *in vivo* half-life is approximately one third of the other IgG isotypes. Another important consideration for immunoadhesins designed to be used as human therapeutics is the number of allotypic variants of the particular isotype. In general, IgG isotypes with fewer serologically-defined allotypes are preferred. For example, IgG-1 has only four serologically-defined allotypic sites, two of which (G1m and 2) are located in the Fc region; and one of these sites G1m1, is non-immunogenic. In contrast, there are 12 serologically-defined allotypes in IgG-3, all of which are in the Fc region; only three of these sites (G3m5, 11 and 21) have one allotype which is nonimmunogenic. Thus, the potential immunogenicity of a $\gamma 3$ immunoadhesin is greater than that of a $\gamma 1$ immunoadhesin.

In designing the OB-Ig immunoadhesins of the present invention regions that are not required for receptor binding, the structural integrity (e.g. proper folding) and/or biological activity of the molecule, may be deleted. In such structures, it is important to place the fusion junction at residues that are located between domains, to avoid misfolding. With respect to the parental immunoglobulin, a useful joining point is just upstream of the cysteines of the hinge that form the disulfide bonds between the two heavy chains. In a frequently used design, the codon for the C-terminal residue of the "adhesin" (OB) part of the molecule is placed directly upstream of the codons for the sequence DKTHTCPPCP of the IgG1 hinge region.

OB-Ig immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the OB portion in-frame to an Ig cDNA sequence. However, fusion to genomic Ig fragments can also be used (see, e.g. Gascoigne *et al.*, *Proc. Natl. Acad. Sci. USA* 84, 2936-2940 [1987]; Aruffo *et al.*, *Cell* 61, 1303-1313 [1990]; Stamenkovic *et al.*, *Cell* 66, 1133-1144 [1991]). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequence from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. Murine OB cDNA can, for example, be obtained by PCR from a mouse adipose tissue cDNA library (Clontech), using primers designed based on the sequence of Zhang *et al.* Human OB cDNA can be obtained in a similar manner. Alternatively, the mouse OB gene can be used as a probe to isolate human adipose tissue cDNA clones (Clontech), e.g. from a λ gt11 library, as described by Zhang *et al.* The cDNAs encoding the 'adhesin' and the Ig parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells. For

expression in mammalian cells pRK5-based vectors (Schall *et al.*, Cell **61**, 361-370 [1990]), pRK7-vectors and CDM8-based vectors (Seed, Nature **329**, 840 [1989]) are preferred. (pRK7 is identical to pRK5 except that the order of the endonuclease restriction sites in the polylinker region between ClaI and HindIII is reversed. See U.S. Patent No. 5,108,901 issued 28 April 1992.). The exact junction can be created by removing the extra sequences between the designed junction codons using oligonucleotide-directed deletional mutagenesis (Zoller and Smith, Nucleic Acids Res. **10**, 6487 [1982]; Capon *et al.*, Nature **337**, 525-531 [1989]). Synthetic oligonucleotides can be used, in which each half is complementary to the sequence on either side of the desired junction; ideally, these are 36 to 48-mers. Alternatively, PCR technique can be used to join the two parts of the molecule in-frame with an appropriate vector.

Immunoadhesins can be expressed efficiently in a variety of host cells, including myeloma cell lines, Chinese Hamster ovary (CHO) cells, monkey COS cells, human embryonic kidney 293 cells, and baculovirus infected insect cells. In these systems, the immunoadhesin polypeptides are assembled and secreted into the cell culture medium. Yeasts, e.g. Saccharomyces cerevisiae, Pichia pastoris, etc., and bacterial cells, preferably E. coli, can also be used as hosts. The OB-immunoglobulin chimeras can be expressed in yeast, for example, similarly to the process described for the expression of the OB proteins by Leiber *et al.*, Crit. Rev. Food Sci. Nutr. **33**, 351 (1993); Friedman and Leibel, Cell **69**, 217 (1992); and Beavis and Chait, Proc. Natl. Acad. Sci. USA **87**, 6873 (1990). Thus, the coding sequences can be subcloned into a yeast plasmid, such as the yeast expression plasmid pPIC.9 (Invitrogen). This vector directs secretion of heterologous proteins from the yeast into the culture media. According to Halaas *et al.*, *supra*, expression of mouse and human OB genes in Saccharomyces cerevisiae transformed with this vector yields a secreted 16-kD protein, which is an unprocessed OB protein lacking the signal sequence. Expression of the mouse or human OB-immunoglobulin chimeras in E. coli can, for example, be performed on the analogy of the procedure described by Halaas *et al.*, *supra*. The coding sequences of mouse and human OB-immunoglobulin chimeras can be subcloned into the PET15b expression vector (Novagen) and expressed in E. coli (BL21 (DE3)pLYsS) through use of the T7 E. coli RNA polymerase system. Alternatively, the fusion protein can be expressed in E. coli by inserting the coding sequence in frame with the secretion sequence of the E. coli heat stable enterotoxin II, downstream of the E. coli alkaline phosphatase promoter (Chang *et al.*, Gene **55**, 189-96 [1987]).

The choice of host cell line for the expression of OB-Ig immunoadhesins depends mainly on the expression vector. Another consideration is the amount of protein that is required. Milligram quantities often can be produced by transient transfections. For example, the adenovirus E1A-transformed 293 human embryonic kidney cell line can be transfected transiently with pRK5- and pRK7-based vectors by a modification of the calcium phosphate method to allow efficient immunoadhesin expression. This method is illustrated in the examples. CDM8-based vectors can be used to transfect COS cells by the DEAE-dextran method (Aruffo *et al.*, Cell **61**, 1303-1313 [1990]; Zettmeissl *et al.*, DNA Cell Biol. (US) **9**, 347-353 [1990]). If larger amounts of protein are desired, the immunoadhesin can be expressed after stable transfection of a host cell line. For example, a pRK5- or pRK7-based vector can be introduced into Chinese hamster ovary (CHO) cells in the presence of an additional plasmid encoding dihydrofolate reductase (DHFR) and conferring resistance to G418. Clones resistant to G418 can be selected in culture; these clones are grown in the presence of increasing levels

of DHFR inhibitor methotrexate; clones are selected, in which the number of gene copies encoding the DHFR and immunoadhesin sequences is co-amplified. If the immunoadhesin contains a hydrophobic leader sequence at its N-terminus, it is likely to be processed and secreted by the transfected cells. The expression of immunoadhesins with more complex structures may require uniquely suited host cells; for example, components such as light chain or J chain may be provided by certain myeloma or hybridoma cell hosts [Gascoigne *et al.*, 5 1987, *supra*; Martin *et al.*, *J. Virol.* 67, 3561-3568 (1993)].

The expression of immunoadhesins with more complex oligomeric structures may require uniquely suited host cells; for example, components such as light chain or J chain may be provided by certain myeloma or hybridoma cell hosts (Gascoigne *et al.*, *supra*; Martin *et al.*, *J. Immunol.* 67, 3561-68 [1993]).

10 Immunoadhesins can be conveniently purified by affinity chromatography. The suitability of protein A as an affinity ligand depends on the species and isotype of the immunoglobulin Fc domain that is used in the chimera. Protein A can be used to purify immunoadhesins that are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains [Lindmark *et al.*, *J. Immunol. Meth.* 62, 1-13 (1983)]. Protein G is recommended for all mouse isotypes and for human $\gamma 3$ [Guss *et al.*, *EMBO J.* 5, 1567-1575 (1986)]. The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrene-divinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. The conditions for binding an immunoadhesin to the protein A or G affinity column are dictated entirely by the characteristics of the Fc domain; that is, its species and isotype. Generally, when the proper ligand is chosen, efficient binding occurs directly from unconditioned culture fluid. One distinguishing 20 feature of immunoadhesins is that, for human $\gamma 1$ molecules, the binding capacity for protein A is somewhat diminished relative to an antibody of the same Fc type. Bound immunoadhesin can be efficiently eluted either at acidic pH (at or above 3.0), or in a neutral pH buffer containing a mildly chaotropic salt. This affinity chromatography step can result in an immunoadhesin preparation that is >95% pure.

Other methods known in the art can be used in place of, or in addition to, affinity chromatography on 25 protein A or G to purify immunoadhesins. Immunoadhesins behave similarly to antibodies in thiophilic gel chromatography [Hutchens and Porath, *Anal. Biochem.* 159, 217-226 (1986)] and immobilized metal chelate chromatography [Al-Mashikhi and Makai, *J. Dairy Sci.* 71, 1756-1763 (1988)]. In contrast to antibodies, however, their behavior on ion exchange columns is dictated not only by their isoelectric points, but also by a charge dipole that may exist in the molecules due to their chimeric nature. Microheterogeneity of charge can also be a factor for immunoadhesins in which the adhesin portion of the molecule is glycosylated and contains 30 sialic acid. A specific purification protocol is described in the examples.

Results with the numerous immunoadhesins produced so far show that the fusion of the adhesin portion to an Fc region usually does not perturb the folding of the individual domains. Both the adhesin and the immunoglobulin regions appear to fold correctly, and the Fc portion retains many of the effector functions that 35 are characteristic of antibodies, such as binding to Fc receptors.

Methods generally applicable for the construction, expression and purification of immunoadhesins are described, for example, in U.S. Patent Nos. 5,225,538 (issued 6 July 1993) and 5,455,165 (issued 30 October 1995), the disclosures of which are hereby expressly incorporated by reference. Immunoadhesin construction,

expression, purification and various immuno adhesins designs are also described in the review articles by Ashkenazi and Chamow, Methods in Enzymology 8, 104-115 (1995), and Peach and Linsley, Methods in Enzymology 8, 116-123 (1995), the disclosures of which, along with the references cited therein, is hereby expressly incorporated by reference.

5 C. Other long half-life OB derivatives

Other derivatives of the OB proteins, which possess a longer half-life than the native molecules comprise the OB protein or an OB-immunoglobulin chimera, covalently bonded to a nonproteinaceous polymer. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e., a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or *in vitro* methods are
 10 useful, as are polymers which are isolated from native sources. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinyl alcohol and polyvinylpyrrolidone. Particularly useful are polyalkylene ethers such as polyethylene glycol (PEG), polyalkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronic); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose,
 15 fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g. polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextran sulfate, dextran, dextrans, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, e.g. hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol;
 20 heparin or heparan. The polymer prior to cross-linking need not be, but preferably is, water soluble, but the final conjugate must be water soluble. In addition, the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if it is intended to be administered by such routes.

Preferably the polymer contains only a single group which is reactive. This helps to avoid cross-linking
 25 of protein molecules. However, it is within the scope herein to optimize reaction conditions to reduce cross-linking, or to purify the reaction products through gel filtration or chromatographic sieves to recover substantially homogenous derivatives.

The molecular weight of the polymer may desirably range from about 100 to 500,000, and preferably is from about 1,000 to 20,000. The molecular weight chosen will depend upon the nature of the polymer and
 30 the degree of substitution. In general, the greater the hydrophilicity of the polymer and the greater the degree of substitution, the lower the molecular weight that can be employed. Optimal molecular weights will be determined by routine experimentation.

The polymer generally is covalently linked to the OB protein or to the OB-immunoglobulin chimeras through a multifunctional crosslinking agent which reacts with the polymer and one or more amino acid or sugar
 35 residues of the OB protein or OB-immunoglobulin chimera to be linked. However, it is within the scope of the invention to directly crosslink the polymer by reacting a derivatized polymer with the hybrid, or via versa.

The covalent crosslinking site on the OB protein or OB-Ig includes the N-terminal amino group and epsilon amino groups found on lysine residues, as well as other amino, imino, carboxyl, sulfhydryl, hydroxyl or other hydrophilic groups. The polymer may be covalently bonded directly to the hybrid without the use of a multifunctional (ordinarily bifunctional) crosslinking agent. Covalent binding to amino groups is accomplished by known chemistries based upon cyanuric chloride, carbonyl diimidazole, aldehyde reactive groups (PEG alkoxide plus diethyl acetal of bromoacetaldehyde; PEG plus DMSO and acetic anhydride, or PEG chloride plus the phenoxide of 4-hydroxybenzaldehyde, succinimidyl active esters, activated dithiocarbonate PEG, 2,4,5-trichlorophenylchloroformate or P-nitrophenylchloroformate activated PEG.). Carboxyl groups are derivatized by coupling PEG-amine using carbodiimide.

Polymers are conjugated to oligosaccharide groups by oxidation using chemicals, e.g. metaperiodate, or enzymes, e.g. glucose or galactose oxidase, (either of which produces the aldehyde derivative of the carbohydrate), followed by reaction with hydrazide or amino derivatized polymers, in the same fashion as is described by Heitzmann *et al.*, P.N.A.S., 71, 3537-41 (1974) or Bayer *et al.*, Methods in Enzymology 62, 310 (1979), for the labeling of oligosaccharides with biotin or avidin. Further, other chemical or enzymatic methods which have been used heretofore to link oligosaccharides are particularly advantageous because, in general, there are fewer substitutions than amino acid sites for derivatization, and the oligosaccharide products thus will be more homogenous. The oligosaccharide substituents also are optionally modified by enzyme digestion to remove sugars, e.g. by neuraminidase digestion, prior to polymer derivatization.

The polymer will bear a group which is directly reactive with an amino acid side chain, or the N- or C-terminus of the polypeptide linked, or which is reactive with the multifunctional cross-linking agent. In general, polymers bearing such reactive groups are known for the preparation of immobilized proteins. In order to use such chemistries here, one should employ a water soluble polymer otherwise derivatized in the same fashion as insoluble polymers heretofore employed for protein immobilization. Cyanogen bromide activation is a particularly useful procedure to employ in crosslinking polysaccharides.

"Water soluble" in reference to the starting polymer means that the polymer or its reactive intermediate used for conjugation is sufficiently water soluble to participate in a derivatization reaction.

"Water soluble" in reference to the polymer conjugate means that the conjugate is soluble in physiological fluids such as blood.

The degree of substitution with such a polymer will vary depending upon the number of reactive sites on the protein, whether all or a fragment of the protein is used, whether the protein is a fusion with a heterologous protein (e.g. an OB-immunoglobulin chimera), the molecular weight, hydrophilicity and other characteristics of the polymer, and the particular protein derivatization sites chosen. In general, the conjugate contains about from 1 to 10 polymer molecules, while any heterologous sequence may be substituted with an essentially unlimited number of polymer molecules so long as the desired activity is not significantly adversely affected. The optimal degree of cross-linking is easily determined by an experimental matrix in which the time, temperature and other reaction conditions are varied to change the degree of substitution, after which the ability of the conjugates to function in the desired fashion is determined.

The polymer, e.g. PEG, is cross-linked by a wide variety of methods known *per se* for the covalent modification of proteins with nonproteinaceous polymers such as PEG. Certain of these methods, however, are not preferred for the purposes herein. Cyanuronic chloride chemistry leads to many side reactions, including protein cross-linking. In addition, it may be particularly likely to lead to inactivation of proteins containing 5 sulfhydryl groups. Carbonyl diimidazole chemistry (Beauchamp *et al.*, Anal Biochem. 131, 25-33 (1983)) requires high pH (>8.5), which can inactivate proteins. Moreover, since the "activated PEG" intermediate can react with water, a very large molar excess of "activated PEG" over protein is required. The high concentrations of PEG required for the carbonyl diimidazole chemistry also led to problems in purification, as both gel filtration chromatography and hydrophilic interaction chromatography are adversely affected. In addition, the high 10 concentrations of "activated PEG" may precipitate protein, a problem that *per se* has been noted previously (Davis, U.S. Patent No. 4,179,337). On the other hand, aldehyde chemistry (Royer, U.S. Patent No. 4,002,531) is more efficient since it requires only a 40-fold molar excess of PEG and a 1-2 hr incubation. However, the manganese dioxide suggested by Royer for preparation of the PEG aldehyde is problematic "because of the pronounced tendency of PEG to form complexes with metal-based oxidizing agents" (Harris *et al.*, J. Polym. Sci. Polym. Chem. Ed. 22, 341-52 [1984]). The use of a Moffatt oxidation, utilizing DMSO and acetic anhydride, obviates this problem. In addition, the sodium borohydride suggested by Royer must be used at high pH and has a significant tendency to reduce disulfide bonds. In contrast, sodium cyanoborohydride, which is effective at neutral pH and has very little tendency to reduce disulfide bonds is preferred.

Functionalized PEG polymers to modify the OB protein or OB-Ig chimeras of the present invention 20 are available from Shearwater Polymers, Inc. (Huntsville, AL). Such commercially available PEG derivatives include, but are not limited to, amino-PEG, PEG amino acid esters, PEG-hydrazide, PEG-thiol, PEG-succinate, carboxymethylated PEG, PEG-propionic acid, PEG amino acids, PEG succinimidylsuccinate, PEG succinimidyl propionate, succinimidyl ester of carboxymethylated PEG, succinimidyl carbonate of PEG, succinimidyl esters of amino acid PEGs, PEG-oxycarbonylimidazole, PEG-nitrophenyl carbonate, PEG tresylate, PEG-glycidyl 25 ether, PEG-aldehyde, PEG vinylsulfone, PEG-maleimide, PEG-orthopyridyl-disulfide, heterofunctional PEGs, PEG vinyl derivatives, PEG silanes, and PEG phospholides. The reaction conditions for coupling these PEG derivatives will vary depending on the protein, the desired degree of PEGylation, and the PEG derivative utilized. Some factors involved in the choice of PEG derivatives include: the desired point of attachment (lysine or cysteine), hydrolytic stability and reactivity of the derivatives, stability, toxicity and antigenicity of the 30 linkage, suitability for analysis, etc. Specific instructions for the use of any particular derivative are available from the manufacturer.

The long half-life conjugates of this invention are separated from the unreacted starting materials by gel filtration. Heterologous species of the conjugates are purified from one another in the same fashion. The polymer also may be water-insoluble, as a hydrophilic gel.

35 The conjugates may also be purified by ion-exchange chromatography. The chemistry of many of the electrophilically activated PEG's results in a reduction of amino group charge of the PEGylated product. Thus, high resolution ion exchange chromatography can be used to separate the free and conjugated proteins, and to resolve species with different levels of PEGylation. In fact, the resolution of different species (e.g. containing

one or two PEG residues) is also possible due to the difference in the ionic properties of the unreacted amino acids.

D. The use of the OB-immunoglobulin chimeras and other long half-life derivatives

The OB-immunoglobulin chimeras and other long half-life OB derivatives of the present invention are useful for weight reduction, and specifically, in the treatment of obesity and other disorders associated with the abnormal expression or function of the OB gene. Our studies indicate that the OB-immunoglobulin chimeras and other long half-life OB derivatives, e.g. PEGylated OB, reduce the food intake and increase the energy use of animals treated, and are therefore very effective in reducing the weight of both obese and normal subjects. For testing purposes, the molecules of the present invention may be dissolved in phosphate-buffered saline (PBS) (pH 7.4), and administered by intravenous or subcutaneous injection, or infusion.

The long acting OB-derivatives of the present invention may further be used to treat other metabolic disorders such as diabetes and bulimia. The OB protein has been shown to reduce insulin levels in animals, and could be useful to reduce excessive levels of insulin in human patients. The reduction of insulin levels in obese or non-obese patients (e.g. Type I or II diabetics) could restore or improve the insulin-sensitivity of such patients.

In addition, the long half-life OB-derivatives can be used for the treatment of kidney ailments, hypertension, and lung disfunctions, such as emphysema. The OB protein might also cause a mitogenic response in receptor-bearing tissues, acting as a growth factor for these cells.

Therapeutic formulations of the present invention are prepared for storage by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or PEG.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution.

Therapeutic compositions herein generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of administration is in accord with known methods, e.g. injection or infusion by intravenous, intraperitoneal, etc. routes. Sustained release formulations are also foreseen. Suitable examples of sustained release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. Patent 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (U. Sidman *et al.*, 1983, "Biopolymers" 22 (1): 547-556), poly (2-hydroxyethyl-methacrylate) (R. Langer, *et al.*, 1981, "J. Biomed. Mater. Res." 15: 167-277 and R. Langer, 1982, Chem. Tech." 12: 98-105), ethylene vinyl acetate (R. Langer *et al.*, *Id.*) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988A). Sustained release compositions also include liposomes. Liposomes containing a molecule within the scope of the present invention are prepared by methods known *per se*: DE 3,218,121A; Epstein *et al.*, 1985, "Proc. Natl. Acad. Sci. USA" 82: 3688-3692; Hwang *et al.*, 1980, "Proc. Natl. Acad. Sci. USA" 77: 4030-4034; EP 52322A; EP 36676A; EP 88046A; EP 143949A; EP 142641A; Japanese patent application 83-118008; U.S. patents 4,485,045 and 4,544,545; and EP 102,324A.

Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal therapy.

An effective amount of a molecule of the present invention to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 µg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer a molecule of the present invention until a dosage is reached that provides the required biological effect. The progress of this therapy is easily monitored by conventional assay techniques. If the purpose of the treatment is weight reduction, the therapy is normally continued until a desired body weight is reached.

Non-therapeutic uses of the OB protein-immunoglobulin fusions of the present invention include their use to identify and purify OB receptors. The identification and expression cloning of an OB receptor, using an OB protein-immunoadhesin is described in a Reference Example hereinbelow.

The invention will be further illustrated by the following non-limiting examples.

Example 1

Expression of OB-immunoadhesins

Using protein engineering techniques, the human OB protein was expressed as a fusion with the hinge, CH2 and CH3 domains of IgG-1. DNA constructs encoding the chimera of the human OB protein and IgG-1 Fc domains were made with the Fc region clones of human IgG-1. Human OB cDNA was obtained by PCR from human fat cell cDNA (Clontech Buick-Clone cDNA product). The source of the IgG-1 cDNA was the plasmid pBSSK-CH₂CH₃. The chimera contained the coding sequence of the full length OB protein (amino acids 1-167 in Figure 5) and human IgG-1 sequences beginning at aspartic acid 216 (taking amino acid 114 as the first residue of the heavy chain constant region (Kabat *et al.*, Sequences of Proteins of Immunological

Interest 4th ed. [1987]), which is the first residue of the IgG-1 hinge after the cysteine residue involved in heavy-light chain bonding, and ending with residues 441 to include the CH2 and CH3 Fc domains of IgG-1. There was an insert of codons for three amino acids (GlyValThr) between the OB and IgG-1 coding sequences. If necessary, this short linker sequence can easily be deleted, for example by site directed deletion mutagenesis, to create an exact junction between the coding sequences of the OB protein and the IgG-1 hinge region. The coding sequence of the OB-IgG-1 immunoadhesin was subcloned into the pRK5-based vector pRK5tk-neo which contains a neomycinselectable marker, for transient expression in 293 cells using the calcium phosphate technique (Suva *et al.*, Science 237, 893-896 [1987]). 293 cells were cultured in HAM's : Low Glucose DMEM medium (50:50), containing 10% FBS and 2 mM L-Gln. For purification of OB-IgG-1 chimeras, cells were changed to serum free production medium PS24 the day after transfection and media collected after three days. The culture media was filtered.

The filtered 293 cell supernatant (400 ml) containing recombinant human OB-IgG-1 was made 1 mM in phenylmethylsulfonylfluoride and 2 µg/ml in aprotinin. This material was loaded at 4 °C onto a 1 x 4.5 cm Protein A agarose column (Pierce catalog # 20365) equilibrated in 100 mM HEPES pH 8. The flow rate was 75 ml/h. Once the sample was loaded, the column was washed with equilibration buffer until the A₂₈₀ reached baseline. The OB-IgG-1 protein was eluted with 3.5 M MgCl₂ + 2% glycerol (unbuffered) at a flow rate of 15 ml/h. The eluate was collected with occasional mixing into 10 ml of 100 mM HEPES pH 8 to reduce the MgCl₂ concentration by approximately one-half and to raise the pH. The eluted protein was then dialyzed into phosphate buffered saline, concentrated, sterile filtered and stored either at 4 °C or frozen at -70 °C. The OB-IgG-1 immunoadhesin prepared by this method is estimated by SDS-PAGE to be greater than 90% pure.

Example 2

Animal studies

A. Materials and Methods

OB protein Production -- Murine OB cDNA was obtained by PCR from an adipocyte cDNA library using primers based on the sequence of Zhang *et al.*, *supra*. Mature OB protein (amino acids 22-167) was expressed in *E. coli* by inserting the OB coding sequence in frame with the secretion sequence of the *E. coli* heat-stable enterotoxin II, downstream of the *E. coli* alkaline phosphatase promoter. Chang *et al.*, Gene 55, 189-96 (1987). After cell lysis, the insoluble fraction was solubilized in 8 M urea buffer pH 8.35 in the presence of 25 mM DTT. Reduced OB protein was purified by size exclusion and reverse phase HPLC, then refolded in the presence of glutathione. Refolded OB protein was purified by reverse phase HPLC and analyzed by SDS-PAGE and amino acid and mass spectrometry analyses.

Preparation of PEG-hOB -- The PEG derivatives of the human PB protein were prepared by reaction of hOB purified by reverse phase chromatography with a succinimidyl derivative of PEG propionic acid (SPA-PEG) having a nominal molecular weight of 10 kD, which had been obtained from Shearwater Polymers, Inc. (Huntsville, AL). After purification of the hOB protein by reverse phase chromatography, an approximately 1-2 mg/ml solution of the protein in 0.1% trifluoroacetic acid and approximately 40% acetonitrile, was diluted with 1/3 to 1/2 volume of 0.2 M borate buffer and the pH adjusted to 8.5 with NaOH. SPA-PEG was added to

the reaction mixture to make 1:1 and 1:2 molar ratios of protein to SPA-PEG and the mixture was allowed to incubate at room temperature for one hour. After reaction and purification by gel electrophoresis or ion exchange chromatography, the samples were extensively dialyzed against phosphate-buffered saline and sterilized by filtration through a 0.22 micron filter. Samples were stored at 4°C. Under these conditions, the PEG-hOB resulting from the 1:1 molar ratio protein to SPA-PEG reaction consisted primarily of molecules with one 10 kD PEG attached with minor amounts of the 2 PEG-containing species. The PEG-hOB from the 1:2 molar reaction consisted of approximately equal amounts of 2 and 3 PEGs attached to hOB, as determined by SDS gel electrophoresis. In both reactions, small amounts of unreacted protein was also detected. This unreacted protein can be efficiently removed by the gel filtration or ion exchange steps as needed. The PEG derivatives of the human OB protein can also be prepared essentially following the aldehyde chemistry described in EP 372,752 published June 13, 1990.

Animal Studies -- All manipulations involving animals were reviewed and approved by Genentech's Institutional Animal Care and Use Committee. Seven to eight week-old genetically obese C57Bl/6J-*ob/ob* (*ob/ob*) female mice were purchased from Jackson Labs (Bar Harbor, ME). Lean female mice of the same genetic background (C57Bl/6J) were purchased from Harlan Sprague Dawley (Hollister, CA). Mice were housed in groups 3 - 6 with *ad libitum* access to water and standard mouse chow (Purina 5010; Purina Mills, Richmond, IN) in a temperature-, humidity- and light-controlled (lights on at 06:00h, off at 18:00h) colony room.

Miniosmotic pumps (Alzet model 2002; Alza Corp., Palo Alto, CA) were filled with purified recombinant OB protein (100 µg/kg/day) in sterile phosphate-buffered saline (PBS) or PBS alone under sterile conditions following manufacturer's instructions and incubated overnight in sterile saline at room temperature prior to implantation into mice. Mice were anesthetized with ketamine/xylazine, and miniosmotic pumps were implanted subcutaneously in the midscapular region. Daily subcutaneous injections of purified recombinant OB protein, hOB-IgG-1 fusion protein or PBS were made into the midscapular region of conscious mice. Injections were performed within one hour of lights out. The body weight of each mouse (to the nearest 0.1 gram) and the weight of the food contained in the food bin in each cage (to the nearest 0.1 gram) were recorded within one hour of lights out every one to two days. The data are depicted as the mean ± SEM. The number of animals is as described below and in the Figure legends.

B. Results with continuous subcutaneous infusion of OB protein

Lean female mice were treated with murine OB protein either as a continuous subcutaneous infusion or daily subcutaneous injections. The results are shown in Figure 1. The upper chart shows that the OB protein is significantly more effective in reducing body weight when delivered as a continuous infusion than when the same dose is delivered in the form of daily subcutaneous injections. The bottom chart shows the same difference in the ability of the OB protein to reduce adipose tissue weight.

C. Results with the OB-IgG-1 chimera

Obese female *ob/ob* mice were treated with human OB protein or with the human OB-IgG-1 chimera. The data are shown in Figure 2. The data presented in the top chart demonstrate that the hOB-IgG-1 fusion

protein is more potent than the native hOB protein at reducing body weight, when both proteins are administered similarly by daily subcutaneous infusion. It is noted that the increase in potency would be even more expressed, if the data were converted to molar amounts, as only about one third of the OB-IgG-1 chimera comes from the OB protein. The data further confirm the previous observation that continuous subcutaneous infusion (pump) or the hOB protein is more effective than daily subcutaneous injections (inj) at reducing body weight.

The data shown at the bottom chart of Figure 2 show that the hOB-IgG-1 fusion protein substantially reduced food intake. This result was unexpected as it was assumed that the fusion protein would be too large to cross the blood-brain barrier and exert its effect.

Obese (*ob/ob*) female mice were treated with either hOB or the hOB-IgG-1 chimera by daily subcutaneous injections for 7 days. The data shown in Figure 3 again demonstrate that the chimera is more effective than the native hOB protein at reducing body weight (top) and food intake (bottom).

In a further experiment, obese (*ob/ob*) female mice were treated with either the hOB-IgG-1 fusion protein, native hOB or hCD4-IgG-1 (control) by daily subcutaneous injections for seven days. The results shown in Figure 5 affirm that the hOB-IgG-1 fusion protein is more effective than the native hOB protein at reducing body weight (top and middle panels) and food intake (bottom panel).

D. Results with PEG-hOB

Obese female *ob/ob* mice were treated with human OB protein or with PEG derivatives of human OB. The data are shown in Figure 4. The data presented in the top chart demonstrate that PEG-hOB is more potent than the native hOB protein at reducing body weight, when both proteins are administered similarly by daily subcutaneous infusion.

The data shown at the bottom chart of Figure 4 show that the PEG-hOB proteins were substantially more effective in reducing food intake than unmodified native hOB.

Reference Example

Identification and cloning of an OB receptor

The OB protein-immunoassay of Example 1 was used to detect and expression clone an OB receptor. First, to identify a receptor source, several cell lines were screened with 1 µg/ml OB-IgG-1 fusion by flow cytometry. The detection system which consists of a biotin conjugated secondary antibody followed by streptavidin-phycoerythrin provides a dramatic amplification of the signal and allows the detection of cells expressing low numbers of receptors. Two cell lines, human embryonic kidney 293 and human lung A549 cells were found to bind OB-IgG-1 but not an Flt-4 control immunoassay. Specific binding of OB-IgG-1 to the cells was also demonstrated by the addition of excess of bacterially expressed human OB protein. Addition of 10 µg/ml of human OB completely blocks the binding of OB-IgG-1 to 293 cells.

To isolate a cDNA encoding the OB receptor, COSN cells were transiently transfected with pools of approximately 10⁵ clones of an oligo dT primed 293 cell cDNA library in pRK5B. Transfected cells were enriched by panning on plates coated with an anti-human Fc antibody after incubation with OB-IgG-1. After

three rounds of enrichment, 1 of 30 pools gave rise to OB-IgG-1 mediated adherence of COSN cells to the binding plates which could be competed by human leptin. cDNA clones picked randomly from this third round were transfected in pools of 10-20. Individual clones were finally identified after breaking down one pool of 10 that was scoring positive by panning.

5 Sequence analysis revealed a clone of approximately 5300 bp with an open reading frame encoding a protein of 896 amino acids. The sequence corresponded to a type 1 transmembrane protein with a 22 amino acid long signal peptide, 819 amino acid extracellular domain, 21 amino acid transmembrane domain and a short 34 amino acid intracellular domain. The sequence was found to essentially correspond to the human OB receptor identified and isolated by Tartaglia *et al.*, *supra*, and is identical with a human receptor sequence
10 disclosed in copending application Serial No. 08/585,005 filed January 11, 1996.

 While the invention has been illustrated by way of examples, the scope of the invention is not so limited. It will be understood that further modifications and variations are possible without diverting from the overall concept of the invention. All such modifications are intended to be within the scope of the present invention.

15 All references cited throughout the specification, including the examples, and the references cited therein are hereby expressly incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Genentech, Inc.
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5 Levin, Nancy
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- (ii) TITLE OF INVENTION: OB Protein Derivatives
- (iii) NUMBER OF SEQUENCES: 2
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- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
20 (D) SOFTWARE: WinPatin (Genentech)
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(C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

- 40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7127 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TATTGCTGAT AAATCTGGAG CCGGTGAGCG TGGGTCTCGC GGTATCATTG 6050
CAGCACTGGG GCCAGATGGT AAGCCCTCCC GTATCGTAGT TATCTACACG 6100
ACGGGGAGTC AGGCAACTAT GGATGAACGA AATAGACAGA TCGCTGAGAT 6150
AGGTGCCTCA CTGATTAAGC ATTGGTAACT GTCAGACCAA GTTTACTCAT 6200
15 ATATACTTTA GATTGATTTA AAACCTCATT TTTAATTTAA AAGGATCTAG 6250
GTGAAGATCC TTTTGTATA TCTCATGACC AAAATCCCTT AACGTGAGTT 6300
TTCGTTCCAC TGAGCGTCAG ACCCCGTAGA AAAGATCAA GGATCTTCTT 6350
GAGATCCTTT TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAACCA 6400
CCGCTACCAG CCGTGGTTTG TTTGCCGGAT CAAGAGCTAC CAACTCTTTT 6450
20 TCCGAAGGTA ACTGGCTTCA GCAGAGCGCA GATACCAAAT ACTGTCCTTC 6500
TAGTGTAGCC GTAGTTAGGC CACCACTTCA AGAACTCTGT AGCACC GCCT 6550
ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTGCTG CCAGTGGCGA 6600
TAAGTCGTGT CTTACCGGGT TGGACTCAAG ACGATAGTTA CCGGATAAGG 6650
CGCAGCGGTC GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG 6700
25 CGAACGACCT ACACCGAACT GAGATACCTA CAGCGTGAGC ATTGAGAAAG 6750
CGCCACGCTT CCCGAAGGGA GAAAGGCGGA CAGGTATCCG GTAAGCGGCA 6800
GGGTCGGAAC AGGAGAGCGC ACGAGGAGC TTCCAGGGGG AAACGCTGG 6850
TATCTTTATA GTCCTGTCGG GTTCGCCAC CTCTGACTTG AGCGTCGATT 6900

TTTGTGATGC TCGTCAGGGG GCGGAGCCT ATGGAAAAAC GCCAGCTGGC 6950
 ACGACAGGTT TCCCGACTGG AAAGCGGGCA GTGAGCGCAA CGCAATTAAT 7000
 GTGAGTTACC TCACTCATTG GGCACCCAG GCTTTACACT TTATGCTTCC 7050
 GGCTCGTATG TTGTGTGGAA TTGTGAGCGG ATAACAATTT CACACAGGAA 7100
 5 ACAGCTATGA CCATGATTAC GAATTAA 7127

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 397 amino acids
 (B) TYPE: Amino Acid
 10 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

	Met	His	Trp	Gly	Thr	Leu	Cys	Gly	Phe	Leu	Trp	Leu	Trp	Pro	Tyr	
	1				5					10					15	
	Leu	Phe	Tyr	Val	Gln	Ala	Val	Pro	Ile	Gln	Lys	Val	Gln	Asp	Asp	
15				20						25					30	
	Thr	Lys	Thr	Leu	Ile	Lys	Thr	Ile	Val	Thr	Arg	Ile	Asn	Asp	Ile	
				35						40					45	
	Ser	His	Thr	Gln	Ser	Val	Ser	Ser	Lys	Gln	Lys	Val	Thr	Gly	Leu	
				50						55					60	
20	Asp	Phe	Ile	Pro	Gly	Leu	His	Pro	Ile	Leu	Thr	Leu	Ser	Lys	Met	
				65						70					75	
	Asp	Gln	Thr	Leu	Ala	Val	Tyr	Gln	Gln	Ile	Leu	Thr	Ser	Met	Pro	
				80						85					90	
	Ser	Arg	Asn	Val	Ile	Gln	Ile	Ser	Asn	Asp	Leu	Glu	Asn	Leu	Arg	
25				95						100					105	
	Asp	Leu	Leu	His	Val	Leu	Ala	Phe	Ser	Lys	Ser	Cys	His	Leu	Pro	
				110						115					120	
	Trp	Ala	Ser	Gly	Leu	Glu	Thr	Leu	Asp	Ser	Leu	Gly	Gly	Val	Leu	
				125						130					135	
30	Glu	Ala	Ser	Gly	Tyr	Ser	Thr	Glu	Val	Val	Ala	Leu	Ser	Arg	Leu	
				140						145					150	
	Gln	Gly	Ser	Leu	Gln	Asp	Met	Leu	Trp	Gln	Leu	Asp	Leu	Ser	Pro	
				155						160					165	
	Gly	Cys	Gly	Val	Thr	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	
35				170						175					180	
	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	

	185	190	195
	Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr		
	200	205	210
5	Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe		
	215	220	225
	Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys		
	230	235	240
	Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val		
	245	250	255
10	Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys		
	260	265	270
	Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr		
	275	280	285
15	Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr		
	290	295	300
	Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu		
	305	310	315
	Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu		
	320	325	330
20	Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro		
	335	340	345
	Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu		
	350	355	360
25	Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys		
	365	370	375
	Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser		
	380	385	390
	Leu Ser Leu Ser Pro Gly Lys		
	395	397	

CLAIMS:

1. A long half-life derivative of an OB protein retaining a biological property of a native OB protein.
2. The long half-life derivative of claim 1 capable of reducing body weight and/or food intake in an individual treated.
3. The derivative of claim 1 which is a derivative of a native human OB protein.
4. The derivative of claim 1 which is an OB-immunoglobulin chimera.
5. The derivative of claim 1 which is a native OB protein or an OB-immunoglobulin chimera modified with a nonproteinaceous polymer.
6. The derivative of claim 4 wherein the nonproteinaceous polymer is polyethylene glycol (PEG).
7. A composition for the treatment of a condition associated with the abnormal expression or function of the OB gene, or for eliciting a biological response mediated by an OB receptor, comprising an effective amount of an OB derivative of claim 1.
8. The composition of claim 7 effective for weight and/or appetite reduction.
9. The composition of claim 7 effective in the reduction of elevated insulin levels.
10. A method for the treatment of a condition associated with the abnormal expression or function of the OB gene, or for eliciting a biological response mediated by an OB receptor, comprising administering to an individual to be treated a derivative of claim 1.
11. The method of claim 10 wherein the condition to be treated is selected from the group consisting of obesity, bulimia, and Type I or II diabetes.
12. A method for inducing weight loss or appetite loss in a subject, comprising administering to said subject an effective amount of a derivative of claim 1.
13. A chimeric polypeptide comprising an OB protein amino acid sequence capable of binding to a native OB receptor, linked to an immunoglobulin sequence.
14. The chimeric polypeptide of claim 13 wherein said immunoglobulin sequence is a constant domain sequence.
15. The chimeric polypeptide of claim 14 wherein said OB protein is human.
16. The chimeric polypeptide of claim 15 wherein two OB polypeptide-IgG heavy chain fusions are linked to each other by at least one disulfide bond to yield a homodimeric immunoglobulin-like structure.
17. The chimeric polypeptide of claim 16 wherein at least one of said OB polypeptide-IgG heavy chain fusions is associated with an immunoglobulin light chain.
18. An isolated nucleic acid sequence encoding an OB protein-immunoglobulin fusion.
19. A replicable expression vector comprising the nucleic acid of claim 18.
20. A host cell transformed with the replicable expression vector of claim 19.
21. A process comprising culturing the host cells of claim 16 so as to express the nucleic acid encoding an OB protein-immunoglobulin fusion.

22. The process of claim 21 wherein said host cells are cotransformed with nucleic acid encoding at least two OB protein-immunoglobulin fusions.
23. The process of claim 22 wherein said cells are further transformed with nucleic acid encoding at least one immunoglobulin light chain.
- 5 24. A method of treating a condition associated with the abnormal expression or function of the OB gene or for eliciting a biological response mediated by an OB receptor comprising administering to a patient a therapeutically effective amount of the chimeric polypeptide of claim 13.
25. The method of claim 20 wherein said condition is selected from the group consisting of obesity, bulimia and type I or II diabetes.
- 10 26. A composition for the treatment of obesity comprising an effective amount of a chimeric polypeptide of claim 13 in association with a pharmaceutically acceptable carrier.
27. A method for inducing the growth of cells expressing an OB receptor comprising contacting said cells with the OB derivative of claim 1.

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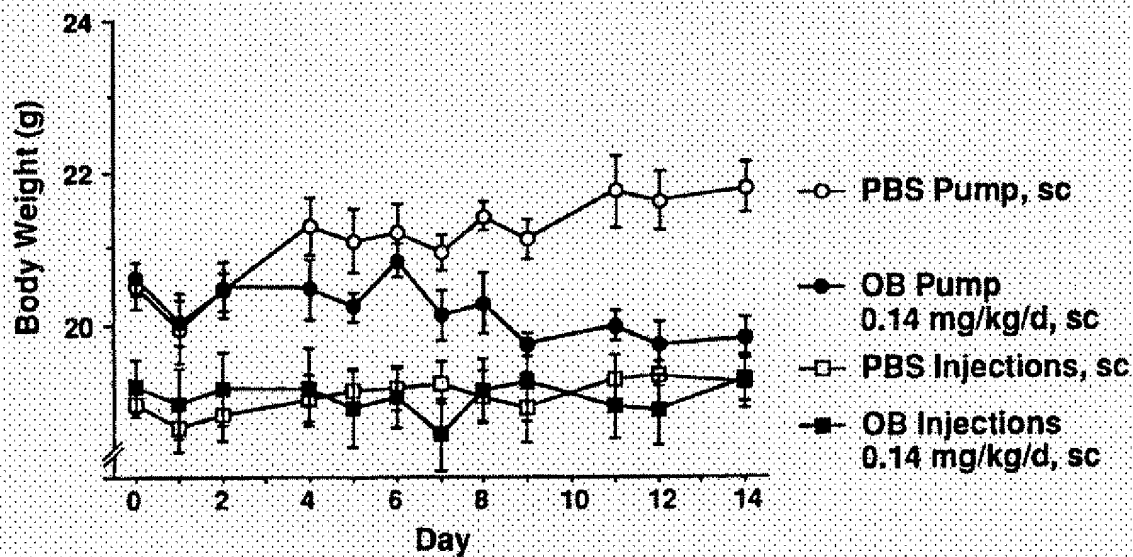


FIG. 1A

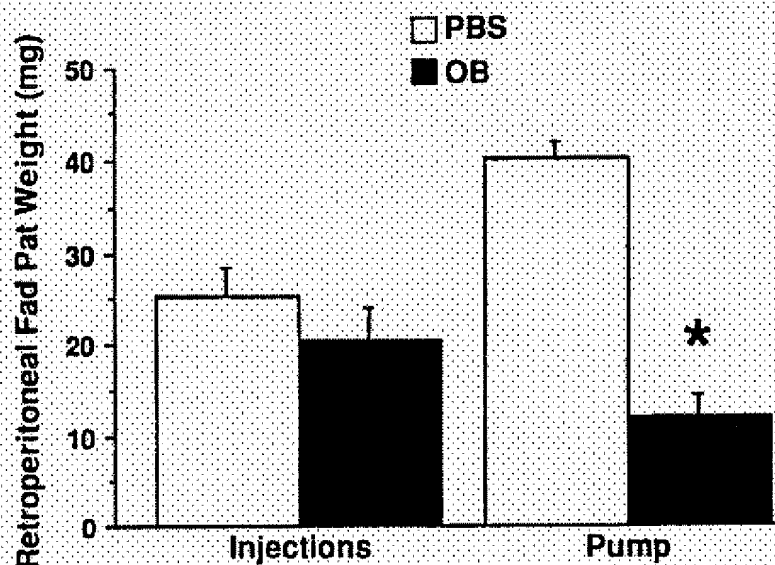


FIG. 1B

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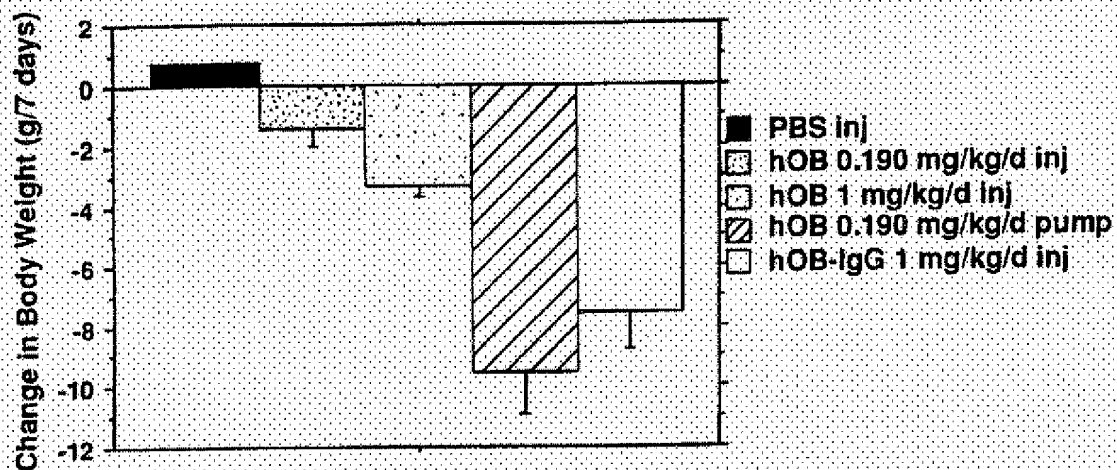


FIG. 2A

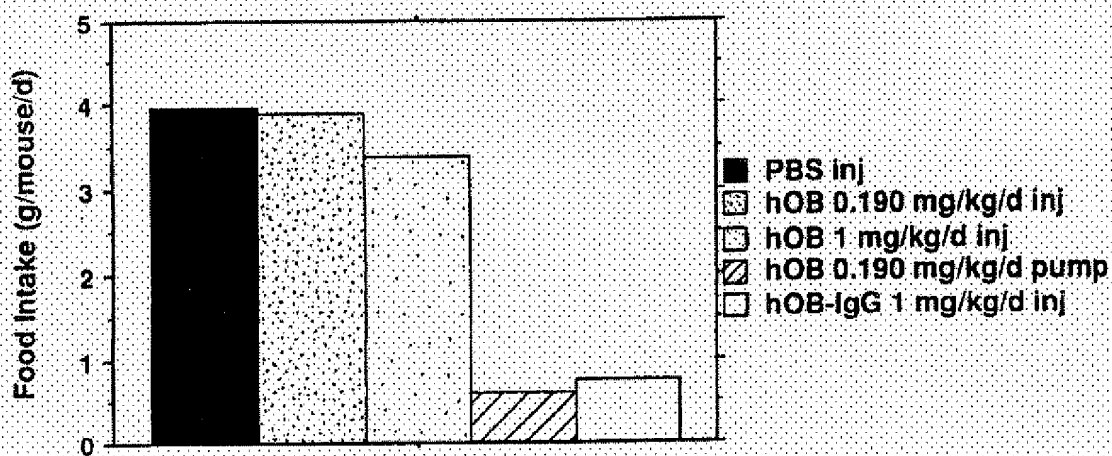


FIG. 2B

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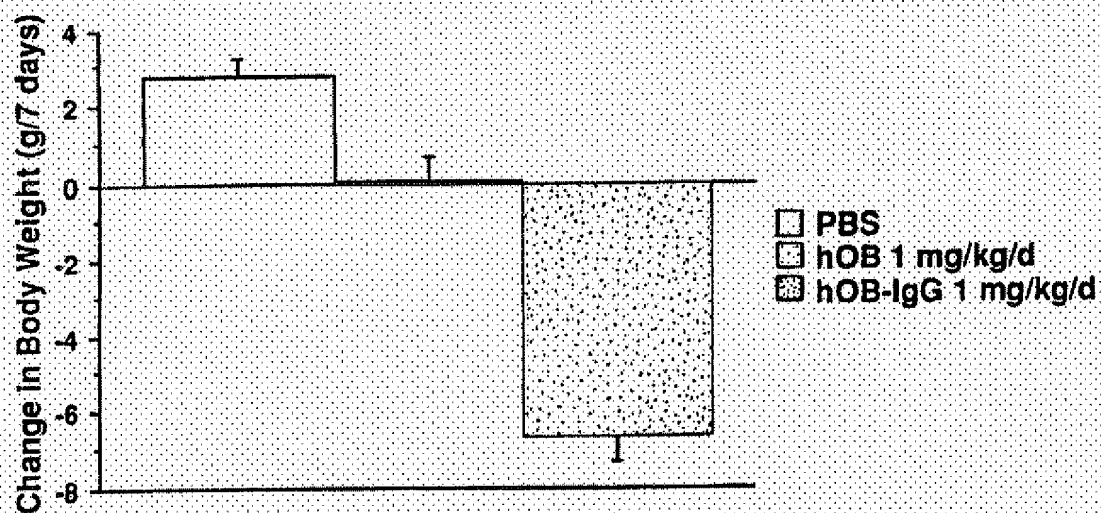


FIG. 3A

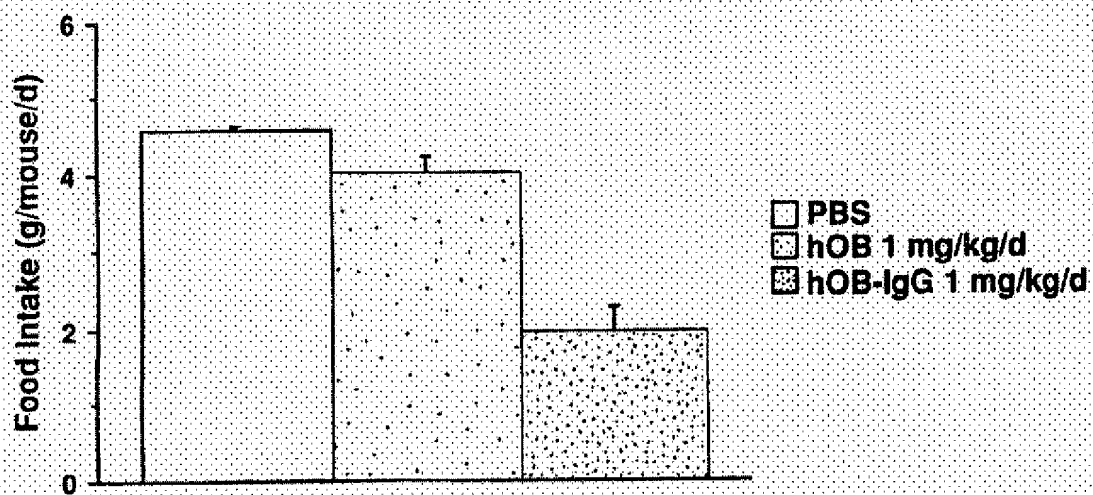


FIG. 3B

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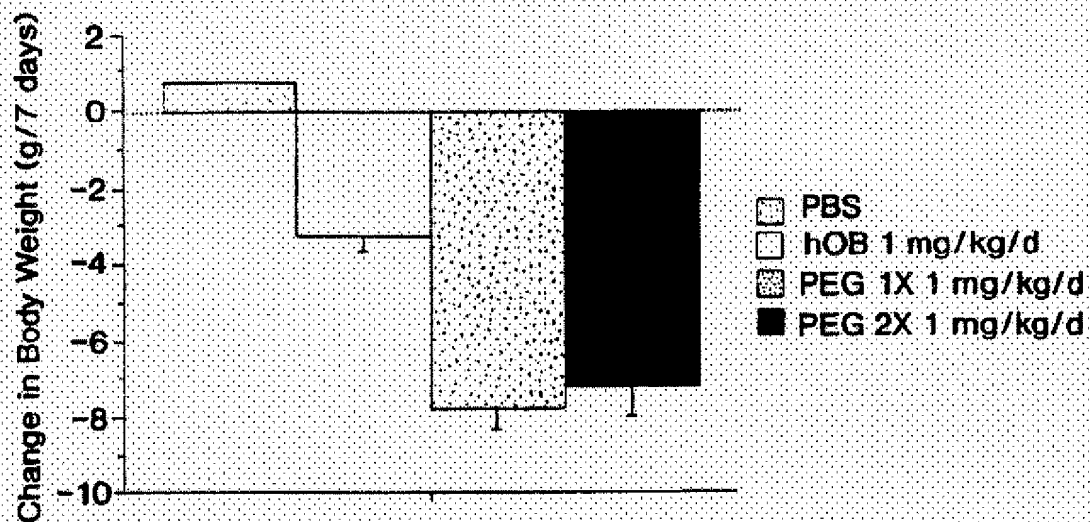


FIG. 4A

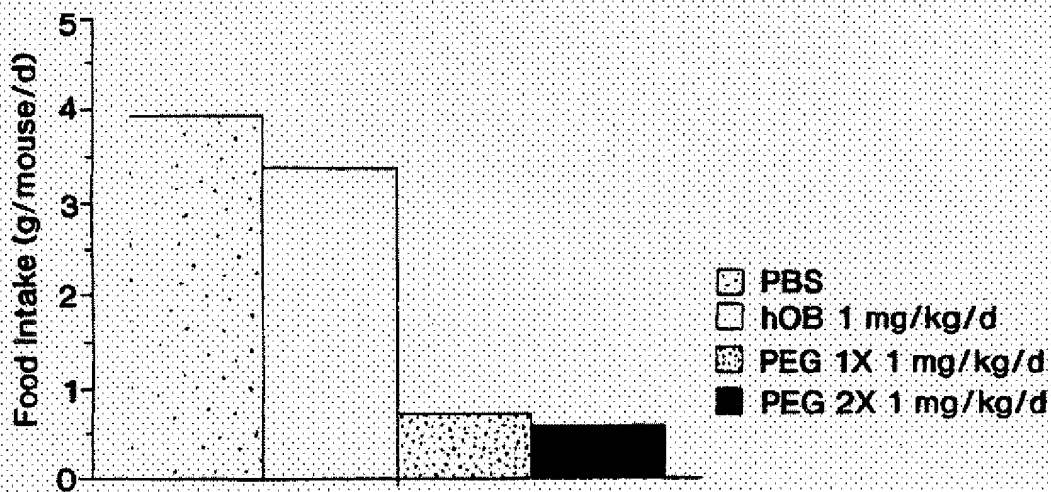


FIG. 4B

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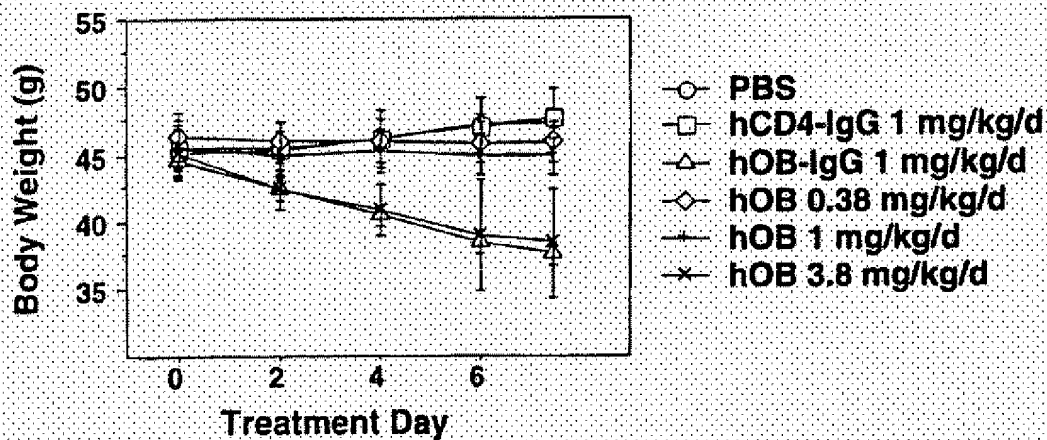


FIG. 5A

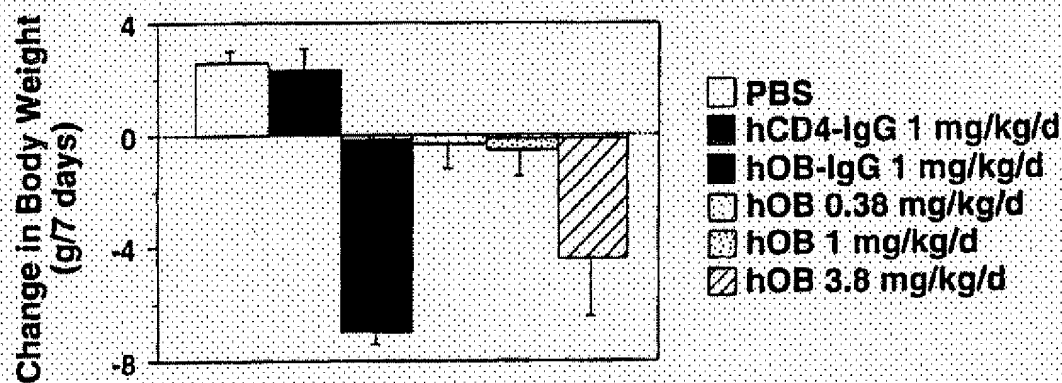


FIG. 5B

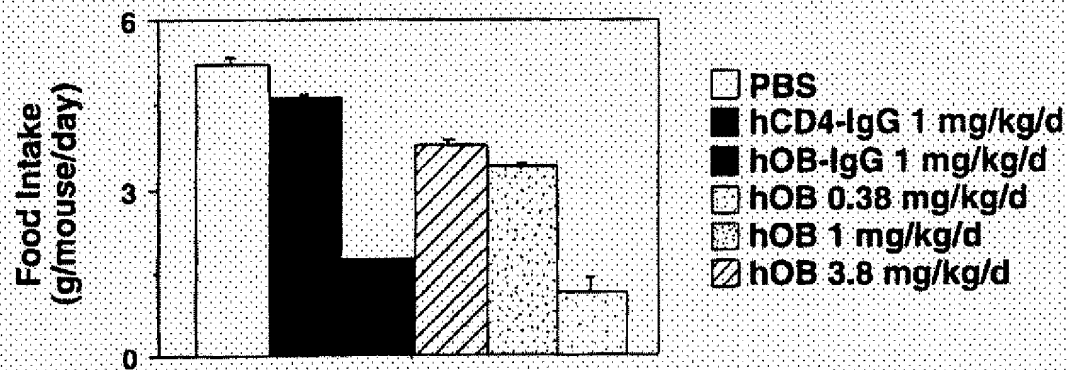


FIG. 5C

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```

ss.PRKStkneo.hob1gc

> sites: std
> length: 7127 (circular)
>
>human OB Clal/BstEII cloning
>CMV enhancer/promoter

      aluI
      sstI
      sacI
      hgiI/II
      hgiAI/asphi
      eclI36II
      bspI286
      bsiHKA1
      bmyI
      banII
      tagI
      rmaI   tru9I
      nciI   mscI
      speI   aei/asnI/vspI
      1 TTGAGCTCG CCCGACATTG ATTATGACT AGTTATTAAT AGTAATCAAT TACGGGGTCA TTAGTTCATA CCCCATATAT GGAGTTCCGC GTTACATNAC
      AAGCTCGAGC GGGCTGTNAC TATTAAGTCA TAAATAATTA TCATTAGTGA ATGCCCCAGT AATCAAGTAT CGGGTATATA CCTCAAGCGG CAATGTATTG

      thal
      fnuDII/mvni
      bstOI
      bshI236I
      acII maeIII
      bslI
      101 TTACGGGTAA TGGCCGGCT GCGTACCGC CCNACGACC CCGCCCATTC ACGTCAATTA TGACGTATGT TCCCATAGTA ACCCAATAG GGACTTTCCA
      AATGCCATT ACCGGGGGA CGGACTGGG GGTGTGCTGG GCGGGTAC TGCAGTTATT ACTGCATACA AGGTATCAT TCGGTTATC CCTGAAAGGT

      maeII
      hiniI/acyI
      ahaII/bsaHI
      aatII
      201 TTACGTCTCA TGGTGGAGT ATTACGGTA AACTGCCAC TTGGCAGTAC ATCAAGTGA TCATATGCCA ACTACGCCGC CTATGACGT CAATGACGGT
      AACTGCAGT ACCCACTCA TAAATGCCAT TTGAGGGTG AAGCGTCATC TAGTTCATCT AGTATACGCT TCATCGCGG GATAACTGCA GTTACTGCCA

      maeII
      hiniI/acyI
      ahaII/bsaHI
      aatII
      rsaI
      csp6I
      ndeI
      csp6I
      bglI
      rsaI
      csp6I

```

FIG. 6A

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```

acrfI      nlaIII      styI      ncoI      dsai hphI acII
mvaI
ecorII
acII
bgII dsav
sau96I bstNI
haeIII/paiI
asuI apyI(dcm+)      bsrI nlaIII
301 AAATGGGCG CTGGCATTA TGCCAGTAC ATGACCTTAT GGCACCTTTC TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATCC
TTTACCUGGC GGACCGTAAT ACCGTCATG TACTGCAATA CCTGAAAGG ATCAACCTGC ATGTAGATGC ATAAACAGTA GCGATAATGG TACCACACG
rsal      maeII      hinfI/acyI      nlaIV      hgiCI
csp6I      ahaII/bsaHI      aatII      banI
401 GGTATTGGCA GTACATCAAT GGGGTGGAT AGCGTTTGA CTCACGGGA TTTCGAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTCGCACCA
CCAAACCGT CATGTAGTTA CCGCACCTA TCGCCAACT GAGTCCCT AGGTGCGTA ACTGCACITTA CCTCAACA AACCCTGCT
alul      astI      sacI      hgiII      hgiAI/aspHI
ecII36II
bsp1286
bslHKAI
bmyI
501 AAATCAACGG GACTTCCAA ATGTGCTAA CACTCCGC CCATTGCGG AATGGGCGG TAGGGTGTG CCGTGGAGG TCTATATAAG CAGAGCTGCT
TTTAGTTGCC CTGAAGGT TTACAGCAT GTGAGCGCG GTAACTGG TTTACCGCC ATCGCACAT GGCACCTCC AGATATATTC GTCTCGAGCA
rsal      csp6I      mnlI
maeIII acII      hgaI      acII

```

FIG. 6B

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```

haeIII/palI
McrI
esgI/xbaIII/ecI XI
EaeI
CfrI
fnu4HI
acII
thai
fnuDII/mvniI
sau96I          sacII/sstII
                    nspBII
                    kspI  scrFI
                    dsai  nciI
                    bglI  bslI  mspI
                    sau3AI mniI bstUI
                    mboI/ndelI{dam-} hpall
                    dpnI{dam+} bsaJI dsav
                    dpnII{dam-} bsh1236I
                    aluI{dam-} acII  caulI
501 TTAGTGAACC GTGATGCG CATCCAGCT GTTTGACCT CCATAGAAGA CACCGGACG CCGCGGCGG GAACTGTGCA
    AATCACTGG CAGCTAGCG GACCTGCG GTAGGTGCG CAAACTGCA GGTATCTTCT GTGGCTGCG GTAGGTGCG CCGCGGCGG CTTGCCACCT
    "Begin RNA

tflI
acII
thai hinfI
fnuDII/mvniI
bstUI
bsh1236I
701 TTGCAACCGG GATTCCCGT GCCAAGAGTG ACGTAAGTAC CGCTATAGA GTCTATAGC CCACCGCTT GCCTTCGTTA GAACCGGCT ACAATTATA
    AACCTGCGC CTAGGGCGA CGGTTCTAC TGCATTCTG TGCATATCT CACATATCG GTGCGCGA CCGAAGCAAT CTTGCGCGA TGTATATAT
    "sp6 promoter

```

FIG. 6C

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```

sau96I
avaII
asuI
scrFI
mvaI
ecorII
dsav
bstNI
apyl(dcm+)
bglI bsaJI
801 CATACCTTAT TGTATCATAC ACATACGATT TAGGTGACAC TATAGAAATAA CATCCAGTTT GCCTTCTCT CCACAGGTGT CCACTCCAG GTCCAACTGC
GTATTGGAT ACATAGTATG TGTATGCTAA ATCCACTGTG ATATCTTATT GTAGTGAAA CGGAAGAGA GGTGTCCACA GGTGAGGTG CAGTTTGACG
~sp6 RNA start
maeiII
hphI scrI foki
3'
ppu10I tflI sau96I
mnlI taqI nsiI/avaII hlnII haeIII/palI bsp1286
bsaJI clai/bsp106 nlaIV acII asuI aluI bspI foki
901 ACTTCGGTTC TATGATATG CATTGGGAA CCGTGTGGG ATTCTGTGG CTTTGGCCCT ATCTTTCTA TGTCCAAGCT GTGCCNTCC AAAAGTCCA
TGGAGCCAG ATAGCTATAC GTACCCCTT CCGACACCC TAAGACACC GAACCGGA TAGAAGAT ACAGTTTGA CACGGTAGG TTTTTCAGGT
1 Mat HistpGlyt hrLeuCysGI yPheLeuTrp LeuTrpProt yrLeuPheTy rValGlnAla ValProIles InLyValGln
~cloning linker ~human OB start
sau3AI
mbol/ndelII(dam-)
dpsI(dam+)
scrFI
mvaI
ecorII
dsav
bstNI
apyl(dcm+)
hphI dpsI(dam-)
mnlI muni aluII(dam-) bsmAI
1001 AGATGACACC AAAACCTTCA TCAAGACAT TGTACACAGG ATCAATGACA TTTCACACAC CCAGTCAGTC TCCTCCAAAC AGAAGTCAC CGGTTTGAC
TGTACTGTGG TTTCGGAGT AGTTCGTGA ACAGTGTCC TAGTACTGT AAAGTGTCTG CCGTACATCAG ACAGAGTTTG TCTTCAGTG GCCAAACCTG
29 AspAspThr LysThrLeuX leLysThrII eValThrArg tLeAsnAspI leSerHisThr rGlnSerVal SerSerLysG InLyValTh rGlyLeuAsp

```

FIG. 6D

[illegible]

FIG. 6

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```

scrFI      eam1105I
mvaI       sau96I
ecorII
dsav
bstNI
belI
bsaJI      hphI      nlaIII
          ddel apyI[dcmt+] maeII      nspl      nspl
          mnlI bsaJI acII bsteII      nsplI      bmyI alwNI
1101 GACCTCAGCC CTGGGTGGCG GGTACCCAC ANAACTACA CATGCCACC GTGCCAGCA CCTGAACTCC TGGGGGACC GTACGTCTTC CTCTGCCCC
CTGAGTGGG CACCACAGCC CAAGTGGT TTTGAGTGT GTACGGGTGG CACCGGTGGT GGACTTGAGG ACCCGCTGG CAGTCAGAGG GNAAGGGGG
162 AspleuSerp roGlycysGI yValThrAsp LysThrAsp LysThrHisT hCysProPr oCysProAla ProGluLeuL euGlyGlyPr oSerValPhe LeuPheProPro
      ^Insertion of a gly
      ^START OF HUMAN I9G1 CN2CH3
          sau96I
          nlaIV
          mspI
          hpaII
          scrFI
          nclI
          dsav
          sauJAJ      avall
          mbol/ndelI[dam-]      nlaIII
          nlaIII      cauII      mnlI      nspl
          rcaI dpaI[dam+]      ddel      nsplI
          bspHI[dam-]      asuI      eco8II      maeII
          mnlI      dpaII[dam-]      bsaJ6I/mstII/sauI
          styI      mnlI      dpaII[dam-]      GGTACATGCC GTGGTGGTGG CAGCTGAGCCA CCAAGACCTT GAGGTGAAGT TCAACTGGTA
          bsaJI      GGAACCCCTC ATGATCTCCC GACCCCTGCA GGTACATGCC GTGGTGGTGG CAGCTGAGCCA CCAAGACCTT GAGGTGAAGT TCAACTGGTA
          GTTTGGGT CCGTGGGGT TACTAGAGG CACTGGGACT CCAAGTGTAC CACCACACC TGCATCTGGT GTCTCTGGGA GTCCAGTCA AGTTGCCAT
196 LysProLy sAspThrLeu MetIleSera rGThrProGI uValThrCys ValValVala spValSerHI sGluaspPro GluValLysP heAsnTrpTyr

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FIG. 6F

FIG. 6G

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aspl          dsal          hphI
hpall         mnlI          scfI          aluI          bsaJI
fnu4HI        pleI          hinfI        nlaIV        mboII        tccTCTACAG  CAAGCTCACC
bbvI          hinfI        ggATCCGAC  GGCTCTTCT  TCCTCTACAG  CAAGCTCACC
CGACCTCACC  CTCTGTTATG  CTCTGCTCT  TCTGCTGCTG  CCGAGGACGA  AGGACATGTC  GTTCGACTGG
329 ValGluTrp GluSerAsnG  LysThrThr  roProValle  waspSerAsp  GlySerPheP  heLeuTyrSe  rLysLeuThr

scfI          nciI          mspI          hpaII          dsav
mboII          nlaIII        ppulOI        nlaIII        mboII          nlaIII
bpuAI          maeII          nlaIII        ppuLOI        nlaIII        mboII          nlaIII
bspMI          fnu4HI        xmiI          bbsI          asp700          nlaIII        sfaMI          mnlI
2001 GTGCACAAGA  GCACGTGGCA  GCAGGCGGAC  GTCTTCTCAT  GTCCCGTGAT  GCATGAGCCT  CTGCACAACC  ACTACACGCA  GAAGAGCCTC  TCCCTGCTC
CAGCTGTTCT  GTTCACACGT  GTTCCCTTG  CAGAGAGTA  CGAGGCACTA  CGTACTCCGA  CAGCTGTTGG  TGAATGGGT  CTTCGCGAG  AGGACACAG
362 ValAspLys  eRaTgTrpG1  nGldGlyAsn  ValPheSerC  ysSerValme  thsGlnAla  LeuHisAsnH  ltyrThrG1  nLysSerLeu  SerLeuSerPro

tagI          tagI          tagI          tagI          tagI          tagI          tagI          tagI          tagI          tagI
sali          pleI          scfI          pleI          scfI          pleI          scfI          pleI          scfI          pleI
rmaI          hincII/hindII  rmaI          hincII/hindII  rmaI          hincII/hindII  rmaI          hincII/hindII  rmaI          hincII/hindII
sau96I          hinfI          pstI          xbaI          hincII/hindII  cfrI          bsaJI          aluI          fnu4HI
haeIII/palI          bspI          aluI          maeI          accI          bspI          aluI          haeIII/palI          aluI
asul          maeI          accI          bspMI          hindIII          hinfI          bspMI          hindIII          accI          asul          bbvI
2101 CCGGTAAATG  AGTCCACCG  CCTTAGATC  GACCTGGAGA  AGCTTCTAGA  GTCCACTGTC  AGAAGCTTGG  CCGCATGGC  CCACCTGTT  TATTCAGCT
CGCATTTAC  TCACCTGCC  GCGATCTCAG  CTGGACGCT  TCGAAGATCT  CAGCTGACG  TCTTCGACG  GCGCTACCG  GGTTCAGCA  ATTAAGTGA
396 GlyLys

maeIII          sfaMI          apol          rmaI          bsaI          maeI
2201 TATAATGGTT  ACAATAAAG  CAATAGCATC  ACANATTCA  CAATAAAGC  ATTTTTC  CTCATCTGA  GTTCTGGTT  GTCCAACTC  ATCAATGTAT
ATATTACCA  TCTTTATTC  GTTATCTAG  TGTTTAACT  GTTATTTCG  TAAAAAAGT  GAGTAAGAT  CAACACCAA  CAGCTTCAG  TAGTTACATA

```

FIG. 6H

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```

sau3AI
mboI/ndeII[dam-]
dpmI[dam+]
dpmI[dam-]
pvuI/bspCI
scri
taqI[dam-] tru9I
clai/bsp106[dam-]
sau3AI msel
mboI/ndeII[dam-]
dpmI[dam+] xmiI
dpmI[dam-] asel/asnI/vsPI bsaJI
nlalII alvi[dam-] asp700 hhaI/cfoI nlalII
2301 CTTATCATGT CTGGATCCAT CCGGAAATTAA TTCCGCCGAC CACCATGGCC TGAATAAACC TGTGAAGAGC GAACCTGGGT AGGTACCTTC TGAGGCCGAA
GAAVAGTACA CACCTAGCTA GGCCTTAATT AAGCCGGGTC GTGGTACCGG ACTTATATGG AGACTTCTC CTTGAACCAA TCCATGGUAG ACTCGGCTT
~ev10 origin

nlalIV
scrFI sfaNI ppu10I auaI
mvaI ecorII dsav bstNI apyI[dcn+]
scrFI ecorII dsav bstNI apyI[dcn+]
sexAI

alul
pvuII
nspBII

nlalIV
scrFI sfaNI ppu10I auaI
mvaI ecorII dsav bstNI apyI[dcn+]
bsaJI nspHI nspHI

nlalIV
scrFI ppu10I
mvaI nsiI/avaIIII
dsav nlalII sphi nspI nspHI
apyI[dcn+]

2401 AGAACCAAGT GTGGATGTG TGTGATGTAG GGTGTGGAA GTCCGAGGC TCCCGAGCG GCGAGAGTAT GCGAAGCATG CATCTCAATT AGTCAGGCAAC
TCTTGCTGCA CACCTTACAC ACAGTCAATC CCACACCTTT CAGCGGTCCG AGCGGTGCTC CGTCTTCATA CGTTGCTGAC GTAGAGTAA TCAGTGTG
nlalIV
scrFI
mvaI ppu10I
ecorII nsiI/avaIIII
dsav nlalII sphi nspI sfaNI
bstNI nspHI nspHI
apyI[dcn+] bsaJI

2501 CAGGTGTGCA AGTCCCGAG GTTCCCGAGC AGGCAGAGT ATGCCAAGCA TGCATCTCAA TTAGTCAGCA ACCATAGTCC CCGCCTTAC TCAGCCCATC
GTCCACACCT TTACGGGCTC CGAGGGGCTC TCCTCTTCA TAGCTTCTGT ACCTAGAGTT AATCAGTCTGT TCGTATCAGG GCGGGGATG AGCGGGGTAG

```

FIG. 6I

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[illegible]

FIG. 6J

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[illegible]

FIG. 6K

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3501	CGGAATATCA TGGTGGAAAA TGGCGCTTT TCTGGATTCA TGGACTCTGG CCGCGTGGGT GTGCGCGACC GCTATCAGGA CATAGCGTTG GCTACCGCGT	mspi hpaII naeI cfrI01 haeIII/palI eaeI tflr hinfI taqI cfrI acII acII	bsII sau96I avaII asuI rsrII/cspI cpoI acII acII	
	GGCTTATAGT ACCACCTTTT ACCGGCGAAA AGACTTAAGT ACCTGACACC GGGCGACCCA CACCGCGCTGG CGATAGTCTT GTATCGGAAC CGATGGGCAC	haeIII/palI eaeI tflr hinfI taqI cfrI acII acII	rsrII/cspI cpoI acII acII	
3601	ATATTGCTCA AGAGCTTGGC GGGGAATGGG CTGACCGCCT CTCTGGTGGT TACGGTATGC CCGCTCCCGA TTGCGACGCC ATGCGCTTCT ATGCGCTTCT	acII fnu4HI haeIII/palI eaeI tflr hinfI taqI cfrI acII acII	bsrBI acII tflr fnu4HI hinfI bbsI sfanI	hlnPI hhal/cfoI acII tflr fnu4HI
	TATTAAGGAT TCTCGAACC GCGCTTACGC GACTGGCGAA GGAGCACGAA ATGCCATAGC GCGAGGGCT ANGCGTGGC TAGCGGAAGA TAGCGGAAGA	acII fnu4HI haeIII/palI eaeI tflr hinfI taqI cfrI acII acII	bsrBI acII tflr fnu4HI hinfI bbsI sfanI	hlnPI hhal/cfoI acII tflr fnu4HI
3701	TCACCGAGTTC TTCTGAGGG GACTCTGGGG TTGGAATAGA CCGACCAACG GACCGCCAAC GACCGCCAAC GAGNTTGGC TTCCACCGCC GCTTCTATG	acII fnu4HI haeIII/palI eaeI tflr hinfI taqI cfrI acII acII	bsrBI acII tflr fnu4HI hinfI bbsI sfanI	hlnPI hhal/cfoI acII tflr fnu4HI
	ACTGCTCAG AGACTGGCC CTGAGACCCC AGCTTTACT GGTCTGTTGC CTGCGGTTG GACGCTAGTG CTCTAAGCT TAGGTGGCGC CGGAGAGTAC	acII fnu4HI haeIII/palI eaeI tflr hinfI taqI cfrI acII acII	bsrBI acII tflr fnu4HI hinfI bbsI sfanI	hlnPI hhal/cfoI acII tflr fnu4HI

FIG. 6M

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hgiJ11 sau96I
 bsp1286 nlaIV
 bmyI haeIII/palI
 scrFI sau96I
 mvaI nlaIV
 ecorII hgiJ11 bell
 dsav ecoO1091/draII sau96I
 bstNI bsp1386 nlaIV
 bsajI bsp120I avall styI
 apyI[dcM+] bmyI ssuI ncoI
 bsajI banII ppuMI dsal
 sau96I xcmI ssuI dsal nlaIV bsaJI
 haeIII/palI fnu4HI fnu4HI
 hphI hphI maeII acII
 bspI ssuI banII bbvI
 4101 CAACCCCCCA GTTCGGTGA AGCCCGAGG CTCGAGCCA ACGTCCGGG GCGAGCCG CCATAGCCAC GGGCCCGT GGTTCGGC GGTTCGGC
 GTTCGGGCTT CAACCCACT TCGGGTCC GACCTGGT TCGAGCCCG CCGTTCGGG GGTTCGGC GGTTCGGC CCAATCCCTG CCGAGCGG

sau96I haeIII/palI
 avall ncoI
 ssuI dsal
 bsrI ddeI bsaJI foki
 4201 ATGGGGAATG GTTTATGGT CGTGGGGGT ATTCTTTTC GGTTCGGT GGTTCGGT CACGACTGA CACGACTGA CACGACTGA TTTTGGATG
 TACCCCTTAC CAATATCCA GACCCCCCA TACAAAACC CCGAGCCG CCGAGCCG GTCGTGACT GACTGCTG TCTGGGTACC AANAACCTAC

scrFI sau96I
 mvaI haeIII/palI
 ecorII fnu4HI fnu4HI
 dsav fnu4HI fnu4HI
 bstNI avall nlaIII batUI mspI
 bsaJI nlaIII mspI bsrI bsaJI hpaII
 apyI[dcM+] acII csp6I hhaI/cfoI cauII fnu4HI
 4301 GCTTCGGCAT GACCCCATG TACTGGGCG ACACGACAC CCGGCTCTG TGGCTGCCA ACACCCCGA CCCCCCAAA CACCCCGCG GATTCCTGGC
 GCGACCCGTA CTTGGGTAC ATGACCGCC CTTGGTCTG CCGCCGAC ACACGACT TGTGGGGCT GGGGTTTTT GGTGGCGCG CTAAAGACCG

FIG. 60

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```

acil
thai
fndIII/mvni
bstUI
acil/satII
haeIII/pali bsh1236I
mcrI nspIII
dsal kspI
bsaJI dsal
hphI eagI/xmaIII/eciII
maeiII eaeI bsaJI
bstEII cfrI acil
TTTGTAATTGG TCACCAACGGC CGAGTTTCGC
AACATAACC AGTGGTGGCC GCTCAAGGCG

mspI
hpaII
acil
fnuIII
bsaJI sfaNI
ATCTGTGCCC CTTCCTGCT GGTACGAGCA CGCCTTTTGT
CGGCGGCTG CTGGAATTGG ACTGATGGG TAGAGACGGG GAGAGACCA CCATGCTCT CGCGAARACA

scrFI alaIV
ncil hqICI
dsav scrFI
cauII mvaI
bsaJI ecorII
bsaJI dsav
bsaJI bstNI
sau96I bsaJI
nlaIV haeIII/pali
avall eaeI
asuI cfrI bsp1286
ppuMI mspI apyI{dcm+}
nlaIV hpaII bayI
eco109I/draII baeI
CGGAGCTCG GCGAGGCGAC CTGTGCTAGC AGTTCATGA TAAAGACAGC AGTCATAGT CCGGCGACCA TAGTCATGCC CGCGCCAC CGGAGGAGCG
CGCTTGGCGC CGTCCCGTG GACAGGATGC TCAAGCTACT ATTCTTCTG TCAGTATCA CCGCCTGCT ATCAGTACGG GCGCGGGTG GCTTTCCTCG
*psk122 sequence

acil
thai
fndIII/mvni
bstUI
acil/satII
haeIII/pali bsh1236I
mcrI nspIII
dsal kspI
bsaJI dsal
hphI eagI/xmaIII/eciII
maeiII eaeI bsaJI
bstEII cfrI acil
TTTGTAATTGG TCACCAACGGC CGAGTTTCGC
AACATAACC AGTGGTGGCC GCTCAAGGCG

hinPI mspI
hhaI/cfoI
thai hpaII
fndIII/mvni
bstUI bsaNI
bsh1236I
fnuIII
acil bceI nlaIII acil bsaI
acil bceI nlaIII acil bsaI
TAGTCATGCC CGCGCCAC CGGAGGAGCG
ATCAGTACGG GCGCGGGTG GCTTTCCTCG

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FIG. 6P

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```

          thal          fnuDII/mvnI          trugI apol tru9I
          msel bstUI msel bsh1236I          sspI
5001 GGGCTATTCT TTGATTTAT AAGGATTTT GCGATTTCG GCTATTGCT TAAAAATGA GCTGATTAA CAAAAATTIA ACGGAAITT TAACAAATA
      CCGGATAAGA AACTAATAA TTCCCTAAA CCGCTAAGC CCGATAACCA ATTITTACT CGACTAATT GTTTTAAAT TGGCITTAA ATTGTTTAT
          haeIII/palI          aluI msel          tru9I          sspI
          maeII          mnlI          haeIII/palI          stuI          haeI          msel
          psp1406I          haeIII/palI          stuI          haeI          msel
          tru9I          msel          tru9I          msel          nlaIII          rcal          msel          bspHI          ddel aatII
          maeII          hnlI/acyI          ahall/bsaHI
5101 TTACGTTA CAATTTATG GTGCGGCTT CCGTATGCG CTATTTTAT AGTTAATGT CATGATAATA ATGGTTTCTT AGAGTICAGG TGGCACTTT
      AATTGCAAT GTTAANTAC CAGTCCGA GCACHTGCG GATAAATA TCCANTTACA GTACTATTAT TACCAAGCA TCTCCAGTCC ACCGTGAAA
          delta 2a
          nlaIV          acil          thal          fnuDII/mvnI          bstUI          bsh1236I          hinPI          hhaI/cfoI
          acil          thal          fnuDII/mvnI          bstUI          bsh1236I          hinPI          hhaI/cfoI
          rcal          bspHI          bsrBI          bsmAI          acil nlaIII          sspI
5201 CCGGGAAATG TCGCGGAAC CCTATTGT TTATTTCT AATACATTC AATATGTAT CCGCTATGA GACAAATACC CTGATTAATG CTTCAATAT
      GCGCTTAC ACGCGCTTG GGGATAACA KATAAAGA TTTATGTAAG TTTATACATA GCGGACTACT CTGTATTGG GACTATTAC GAAGTTATA
          mboII          earI/ksp632I          hphI
5301 ATTGAAAG GAAGATAG AGATTCAAC ATTCCCTGT CCGCTTAT CCGCTTTTG CCGCATTTG CCTCCTGTT TTGCTCACC CAGNAAGCT
      TAACCTTTC CTTCATAC TCATAGTTC TAAAGGACA CCGGATAT GCGAATAAC CCGTAAAC GGAAGGACA AAGCAGTGG GTCTTTGGA
          hqAI/aspHI          bsp1286          bsiHKA1          mboI/ndelI[dam-]          dpmI[dam+]          bmyI          dpmI[dam+]          apalI/snoI          eco57I          hphI          sfanI          mboI[dam-]          alw41/snoI          maeIII          taqI          alwI[dam-]          acil          bstYI/xhoII          mboII
          sau3AI          mboI/ndelI[dam-]          dpmI[dam+]          dpmI[dam+]          bstYI/xhoII          nsp8II          alwI[dam-]          bstYI/xhoII          mboII
5401 GGTCAAGTA AAGHTCTG AGATCACTT GGTGCAACA GTGGCTTACA TCGAAGTGA TCTCAACAGC GGTAGATCC TTGAGAGTTT TCGCCCCGAA
      CCACTTTCAT TTCTAGCAG TTCTAGTCA CCACTGTCT CACCAATGT ACCTTCACCT ACAGTTGTC CCAITCTAGG AACTCTCAA ACGCGGGCTT

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FIG. 6R

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maeII hglAI/asplI
 pspI406I bspI286I tru9I
 xbaI bsiHKA1 mseI
 asp700 bmyI ahaII/draI
 5501 GAACGGTTTC CAATGATGAG CACTTTTAA GFTCTGCTAT GTGCGCGGT ATTATCCGT GATCAGCGG GGCAGAGCA ACTCGGTCC CGCATACACT
 CTTCGCAAG GTTACTACTC GTGAATTT CAAGAGGATA CACCGCGCA TAATAGGCA CTACTCGG CCGTCTCGT TGAGCCAGG CGTATGTGA
 rsaI
 csp6I bsrI
 scaI hphI
 ddeI
 5601 ATTCTCAGAA TGACTTGTT GAGTACTAC CAGTCACAGA AAGCATCTT AGCGATGCA TGACGTAG AGATTATGC AGTGTGCCA TACCATGAG
 TAAGATCTT ACTGAACCA CTCTGAGTG GTCACTGTCT TTTCGTAGA TCGCTACCT ACTGCTATC TCTTATAGC TCACGACGCT ATTGTACTC
 fnu4HI
 sau96I
 avaiI
 sau3AI asuI
 haeIII/palI
 eaeI
 cfrI
 fnu4HI
 acII
 5701 TGATACACT GCGCCMACT TACTTCTGAC AACGATCGA GCACCGNAG AGCTAACGC TTTTTCGAC AACATGCGG ATCATGTAG TCGCTTGT
 ACTATTGTGA CCGCGGTTGA ATGAAGACTG TTGCTAGCTT CTTGCTTCC TCGATTGCGT AAAAAAGTG TTGTACCCG TAGTACATG AGCGNACTA
 mspI
 hpaII
 bsaHI
 nlaIV aluI
 maeII
 accGATGAC CCGAGCTGAA TGAACCTA CCNAGACAG ACCGTGAC CACGATGCA CCGACGATG CCAACACTT CGCGAAGCTA TTACTGCGG
 GCAACCCCTG GCGTGCCTT ACTTCGCTT GGTTCCTGC TCGCATCTG GTGCTAGCT GTGCTAGCT GTTGTGCA CCGCTTGT ATTGTAGCGG
 bcrFI
 nciI
 mspI
 hpaII
 dsav
 caulI
 hinII/acyI
 hgaI
 ahaII/dsaHI
 bcrI mcrI fnu4HI
 acII
 5801 CGTTGGGAC CCGAGCTGAA TGAACCTA CCNAGACAG ACCGTGAC CACGATGCA CCGACGATG CCAACACTT CGCGAAGCTA TTACTGCGG
 GCAACCCCTG GCGTGCCTT ACTTCGCTT GGTTCCTGC TCGCATCTG GTGCTAGCT GTTGTGCA CCGCTTGT ATTGTAGCGG
 maeII
 bspI286I tru9I
 xbaI bsiHKA1 mseI
 asp700 bmyI ahaII/draI
 5501 GAACGGTTTC CAATGATGAG CACTTTTAA GFTCTGCTAT GTGCGCGGT ATTATCCGT GATCAGCGG GGCAGAGCA ACTCGGTCC CGCATACACT
 CTTCGCAAG GTTACTACTC GTGAATTT CAAGAGGATA CACCGCGCA TAATAGGCA CTACTCGG CCGTCTCGT TGAGCCAGG CGTATGTGA
 rsaI
 csp6I bsrI
 scaI hphI
 ddeI
 5601 ATTCTCAGAA TGACTTGTT GAGTACTAC CAGTCACAGA AAGCATCTT AGCGATGCA TGACGTAG AGATTATGC AGTGTGCCA TACCATGAG
 TAAGATCTT ACTGAACCA CTCTGAGTG GTCACTGTCT TTTCGTAGA TCGCTACCT ACTGCTATC TCTTATAGC TCACGACGCT ATTGTACTC
 fnu4HI
 sau96I
 avaiI
 sau3AI asuI
 haeIII/palI
 eaeI
 cfrI
 fnu4HI
 acII
 5701 TGATACACT GCGCCMACT TACTTCTGAC AACGATCGA GCACCGNAG AGCTAACGC TTTTTCGAC AACATGCGG ATCATGTAG TCGCTTGT
 ACTATTGTGA CCGCGGTTGA ATGAAGACTG TTGCTAGCTT CTTGCTTCC TCGATTGCGT AAAAAAGTG TTGTACCCG TAGTACATG AGCGNACTA
 mspI
 hpaII
 bsaHI
 nlaIV aluI
 maeII
 accGATGAC CCGAGCTGAA TGAACCTA CCNAGACAG ACCGTGAC CACGATGCA CCGACGATG CCAACACTT CGCGAAGCTA TTACTGCGG
 GCAACCCCTG GCGTGCCTT ACTTCGCTT GGTTCCTGC TCGCATCTG GTGCTAGCT GTTGTGCA CCGCTTGT ATTGTAGCGG

FIG. 6S

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mspi          bglI
hpaII         sau96I
scrFI         haeIII/paII
          aluI nciI      tru9I   fokI
          rmaI dsav      mseI   bsrI   acII
          maeI caulI     aseI/asnI/vspI  mnlI
5901 AACTACTTAC TGTAGCTTCC CGGCAACCAAT TAATAGACTG CATGAGGCG GATAAGTTG CAGGACCCT TCTGCTCG GCGCTTCCG CTGGCTGGTT
TTGATGAATG AGATCGAAGG GCGTTGTGA ATTATCTGAC CTACTCTCG CTATTCAAC GTCTGTGTGA AGACGGAGC CGGAGAGCC GACCGACCA
          acII
mspi          theI
hpaII         fnuDII/mvnI      sau96I
cfrI01        bstXI
          nlaIV hphI      bsmAI      fnuDII nlaIV
          gsuI/bpmI      bsaI bsh1236I      bbsI bsrI haeIII/paII mnlI
6001 TATTGCTGAT AATGCTGAG CGGCTGAGG TGGTCTCG GGTATCATTTG CAGCACTGG GCGCATGGT AAGCCTCCC GTATCGTAGT TATCTACACG
ATAAGCACTA TTTAGACTC GCGCACTCG ACCGACTCG CCATAGTAA CTTGTGAGC GCGTGAGCC CGGTCTACEA TTGGGAGGG CATAGCATCA ATAGATGTC
          ddeI
          sau3AI nlaIV
          mboI/ndelI[dam-] mnlI
          p1eI      hinfI      fokI      dnmI[dam+] hgiCI      tru9I
          ACGGGAGTC AGGCACTAT GGTATGACGA AATAGACAGA TGGCTGAGT AGCTGCTCA CTGATTAAGC ATTGGTAACT GTGAGACCA GTTTACTCAT
TGGCCTCAG TCGTTGATA CTTACTTCT TTATCTGCT AGGACTCTA TCGGCGAGT GACTAATTCG TAACCATGA CAGTCTGTT CAATGAGTA
          maeIII
          hphI
          rmaI      sau3AI
          sau3AI      mboI/ndelI[dam-]
          mboI/ndelI[dam-]
          dnmI[dam+] dnmI[dam+]
          tru9I dnmI[dam-] dnmI[dam-]
          shalII/draI maeI      alvi[dam-]
          tru9I      bstXI/xhoII bstXI/xhoII      nlaIII      rcaI      maeII
          maeI      alvi[dam-] mboI/dam-]
          shalII/draI      maeI maeI      alvi[dam-] mboI/dam-]
6201 ATATACTTTA GATTGATTTA AACTTCATT TTTAATTAA AAGGATGAG GTGAGATCC TTTTGTATA TCTGAGACC AAAATCCCTT ACGTGGAGTT
TATAGCAAT CTACTAAT TTGAAGTAA AATTAATT TTCTAGATC CACTCTAG AATACTATT AGAGTACTGG TTTAGGGA TTECACTCA
          maeI      tru9I      maeI

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FIG. 6T

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sau3AI
mboI/ndeII[dam-]
dpnI[dam+] sau3AI          thalI
dpnII[dam-] mboI/ndeII[dam-]
bstYI/xhoII dpnI[dam+] fnuDII/vnII
sau3AI alwI[dam-] dpnII[dam-] bstUI
mboI/ndeII[dam-] alwI[dam-] bshI236I
dpnI[dam+] mboII[dam-] hinPI fnu4HI
dpnII[dam-] bstYI/xhoII hhaI/cfoI bbvI
6301 TTGTTGCCAC TGAGCGTCAG ACCCGGTAGA AAGATCAAA GGAICTTCTT TTTCGTGCC GTAATGTGCT GCTTGCAAC AAAAAACCA
AAGCAAGTG ACTCCAGTC TGGCCATCT TTCTAGITT CCTAGAGAA CTCTAGAAA AAAGACGCC CATTAGCGA CGAACGTTG TTTTITGCT
dclI hgaI
sau3AI
mboI/ndeII[dam-]
dpnI[dam+]
dpnII[dam-]
alwI[dam-]
mepI
hpaII
acII nsp8II
hpaII aluI
6401 CCGCTACCAAG CGGTGCTTIG TTGCCGGAT CAGAGCTAC CAAGCTTTT TCCGAAGTA ACTGCTTCA CCAGACGCA GATACCAAT ACTGTGCTC
CGCGATGTC GCACCAAC AACGGCTA GTTCTGATC GTTGAAGAA AGCTTCAT TGACCGAAGT GGTCTGCGT CTATGCTTA TGACAGGAAG
rmaI
maeI
bsrI
maeIII eco37I hhaI/cfoI
6501 TAGGTAGCC GTAGTAGCC CACCACTCA AGAAGCTGT AGCAGCGCT ACATAGCTG CTCGCTAAT CTCGTACCA GTGGTGTCTG CCAGTGGCA
ATCAGATCG CATCAATCG GTGGTCAAGT TCTGAGACA TGTGCGCA TGTATGAGC GAGACGATTA GGACATGT CACCGACGAC GTTCACCGCT
fnu4HI
alwNI bbvI
bsrI fnu4HI
maeIII bbvI bsrI
6601 TAAGTCGTGT CTACCGGT TGACTCAAG AGCATAGTA CCGCATAGG CGCAGCGTC GGCCTGAAC GCGGCTGCT CCACACAGCC CAGCTTGGAG
ATTGAGACA GAATGCGCA ACTGAGTTC TCTATCAAT GGCCTATTCC GGTGCTGAG CCGACTTCC CCGCAGCA GGTGTGTCG GTGCAACCTC
hgaI/aspHI
bspI286
bsiHKA
bsyI
apaLI/snoI
alw44I/snoI aluI

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FIG. 6U

INTERNATIONAL SEARCH REPORT

International Application No.
PC1/US 96/20718

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/16 C07K14/575 A61K38/22 C12N15/70 C12N1/21
/(C12N1/21, C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 97 00319 A (SMITHKLINE BEECHAM PLC ;BROWNE MICHAEL JOSEPH (GB); CHAPMAN CONRAD) 3 January 1997 see page 1, line 31 - line 33; claims; examples	1-4, 7-26
P, X	EP 0 741 187 A (HOFFMANN LA ROCHE) 6 November 1996 see page 9, line 19 - page 11, line 46; claims; examples 19, 20	1-3, 6-12, 26
P, X	WO 96 05309 A (UNIV ROCKEFELLER ;FRIEDMAN JEFFREY M (US); ZHANG YIYING (US); PROE) 22 February 1996 see page 43, line 3 - page 46, line 14; claims	1-3, 6-12, 26

	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search

17 April 1997

Date of mailing of the international search report

14.05.97

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Fax: (+31-70) 340-3016

Authorized officer

Fuhr, C

INTERNATIONAL SEARCH REPORT

Inter- national Application No
PC1/US 96/20718

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>NATURE, vol. 372, no. 6505, 1 December 1994, pages 425-432, XP000602062 YIYING ZHANG ET AL: "POSITIONAL CLONING OF THE MOUSE OBESE GENE AND ITS HUMAN HOMOLOGUE" see the whole document -----</p>	<p>1,10-12, 26</p>

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 20718

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 7-10, 24-25
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 7-10, and 24-25 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/20718

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9700319 A	03-01-97	AU 6011096 A	15-01-97
EP 0741187 A	06-11-96	AU 5197896 A	14-11-96
		CA 2175298 A	06-11-96
		ES 2093593 T	01-01-97
		JP 9003098 A	07-01-97
		NO 961796 A	06-11-96
		PL 314051 A	12-11-96
WO 9605309 A	22-02-96	AU 3329895 A	07-03-96
		CA 2195955 A	22-02-96
		DE 19531931 A	07-03-96
		FI 970656 A	17-02-97
		GB 2292382 A	21-02-96
		JP 9502729 T	18-03-97
		ZA 9506868 A	09-04-96